AD_____

Award Number: DAMD17-03-1-0231

TITLE: Targeting Stat3 with G-Quartet Oligonucleotides in Metastatic Prostate Cancer

PRINCIPAL INVESTIGATOR: Naijie Jing, Ph.D.

CONTRACTING ORGANIZATION: Brigham and Women's Hospital
Boston, Massachusetts 02115

REPORT DATE: April 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2005	Annual (1 Apr 2004 - 31 Mar 2005)

4. TITLE AND SUBTITLE
Targeting Stat3 with G-Quartet Oligonucleotides in Metastatic Prostate Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0231

6. AUTHOR(S)
Naijie Jing, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brigham and Women's Hospital
Boston, Massachusetts 02115

E-Mail: njing@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
This annual report is composed of (1) introduction of the goal of this project and specific aims, (2) body of research accomplishments in PI's group from 4/1/04 to 3/31/05, (3) reportable outcomes, and (4) conclusion of researches from 4/1/04 to 3/31/05. The report demonstrated that during the period from 4/1/04 to 3/31/05, PI group made a significant progress in developing a novel and potent anti-cancer agent, which holds promise for the systemic treatment of prostate cancer.

14. SUBJECT TERMS
G-quartet oligonucleotides, Stat3, prostate cancer, rational drug design, anti-cancer agent

15. NUMBER OF PAGES
28

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

I. Introduction.

Our goal is to develop a novel, potent inhibitor of Stat3 as a therapeutic drug for prostate cancer therapy. In the year 2003, carcinoma of the prostate accounted for an estimated 220,900 new cancer cases and 28,900 deaths, including 30,200 deaths in the United States. Prostate cancer is one of the most frequently diagnosed cancers and the second leading cause of cancer death in American men. Current treatments for androgen-independent prostate cancer have not shown a definitive increase in survival. The treatment options employed for patients with advanced and metastatic prostate cancer are limited. Mounting evidence demonstrates that Stat3 is a critical mediator of oncogenic signaling (1-3) and is active in 82% of prostate cancers (4), 69% of breast cancers (5), 90% of head and neck cancers (HNSCC) (6), 71% of nasopharygeal carcinoma (7) as well as in many other cancers. However, drugs targeting Stat3 have not been used to treat prostate cancer. We propose a novel strategy to inhibit Stat3, which could be very useful in development of prostate cancer therapy, and have developed a G-quartet oligodeoxynucleotide (GQ-ODN), T40214, as a lead compound that preferentially inhibits the DNA-binding activity of Stat3 among STAT protein members such as Stat1, resulting in the suppression of Stat3-regulated genes, *bcl-x* and Mcl-1, in cancer cells (8). We also have constructed a model of GQ-ODN binding onto Stat3 homodimer for rational drug design. The molecular model suggests that the GQ-ODN insert between the two SH2 domains of Stat3 dimer resulting in their destabilization. We further show that T40214 and its derivative T40231 have dramatic *in vivo* effects on prostate cancer growth in nude mice when given by intravenous or intraperitoneal injections, dramatically retarding tumor growth and significantly increase the length of survival time (Appendices 1-2). Also GQ-ODNs greatly increased apoptosis in cancer cells where Stat3 is active but did not induce apoptosis in normal epithelial cells, which indicates the agents using apoptosis to eliminate tumor cells in cancer therapy. Developing a novel and promising treatment for prostate cancer will be beneficial both in terms of single agent treatment and as part of combination therapy. The overall goal of this proposal is to develop a potent inhibitor of Stat3 using T40214 and T40231 as lead compounds for prostate cancer therapy. The Specific Aims designed to achieve these objectives are outlined below.

Specific Aim 1: To develop a potent inhibitor of Stat3 to suppress growth of prostate cancer cells in cell culture.
Specific Aim 2: To develop an effective delivery system and to test the inhibition of growth of prostate tumors for our designed inhibitors *in vivo*.

II. Body of research accomplishments in PI's group from 4/1/04 to 3/31/05.

Publication 1: G-quartet oligonucleotides: a new class of signal transducer and activator of transcription 3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis (Jing et al. *Cancer Research* 64:6003-6009 (2004), Appendix 1).
ABSTREAT: Stat3 is a signaling molecular and oncogene activated frequently in many human malignancies including the majority of prostate, breast and head and neck cancers; yet, no current chemotherapeutic approach has been implemented clinically that specifically targets Stat3. We recently developed G-rich oligodeoxynucleotides, which form intramolecular G-quartet structures (GQ-ODN), as a new class of Stat3 inhibitor. GQ-ODN targeted Stat3 protein directly inhibiting its ability to bind DNA. When delivered into cells using polyethyleneimine (PEI) as vehicle, GQ-ODN blocked ligand-induced Stat3 activation and Stat3-mediated transcription of anti-apoptotic genes. To establish the effectiveness of GQ-ODN as a potential

new chemotherapeutic agent, we systemically administered GQ-ODN (T40214 or T40231) plus PEI or PEI alone (placebo) by tail vein injection into nude mice with prostate and breast tumor xenografts. While the mean volume of breast tumor xenografts in placebo-treated mice increased over 7 fold over 18 days, xenografts in the GQ-ODN-treated mice remained unchanged. Similarly, while the mean volume of prostate tumor xenografts in placebo-treated mice increased 9 fold over 10 days, xenografts in GQ-ODN-treated mice increased by only 2 fold. Biochemical examination of tumors from GQ-ODN-treated mice demonstrated a significant reduction in Stat3 activation, levels of the anti-apoptotic proteins Bcl-2 and Bcl-x_L and a 8-fold increase in the number of apoptotic cells compared to the tumors of placebo-treated mice. Thus, GQ-ODN targeting Stat3 induce tumor cell apoptosis when delivered into tumor xenografts and represent a novel class of chemotherapeutic agents that holds promise for the systemic treatment of many forms of metastatic cancer.

Conclusion 1: This work well accomplished the Specific Aims of PC020407 proposal: (1) to develop a potent inhibitor of Stat3 to suppress growth of prostate cancer cells in cell culture; and (2) to develop an effective delivery system and to test the inhibition of growth of prostate tumors for our designed inhibitors *in vivo*.

Publication 2: Rational drug design of G-quartet DNA as anti-cancer agents (Jing et al. *Current Pharmaceutical Design*, in press (2005), Appendix 2).

ABSTRACT: The ability of certain DNA sequences to form G-quartet structures has been exploited recently to develop novel anti-cancer agents including small molecules that promote G-quartet formation within the c-MYC promoter thereby repressing c-MYC transcription and introducing G-quartet-forming oligodeoxynucleotides (GQ-ODN) into cancer cells resulting in p53-dependent cell cycle arrest and inhibition of DNA replication. GQ-ODNs also have been developed as potent inhibitors of signal transducer and activator of transcription (STAT) 3, a critical mediator of oncogenic signaling in many cancers. This review summarizes the rational design of G-quartet forming DNA drugs as Stat3 inhibitors. Topics that are reviewed include the strategy of structure-based drug design, establishment of a structure-activity relationship, development of a novel intracellular delivery system for G-quartet-forming DNA agents and *in vivo* drug testing to assess the anti-cancer effects of DNA drugs in tumor xenografts. Results to date with GQ-ODN targeting Stat3 are encouraging, and it is hoped that continued pursuit of the methodology outlined here may lead to development of an effective agent for treatment of metastatic cancers, such as prostate and breast, in which Stat3 is constitutively activated.

Conclusion 2: This publication well summarized all work that PI group has done to accomplish the project of PC020407 and partly further completed the Specific Aims: (1) to develop a potent inhibitor of Stat3 to suppress growth of prostate cancer cells in cell culture; and (2) to develop an effective delivery system and to test the inhibition of growth of prostate tumors for our designed inhibitors *in vivo*.

Publication 3. Targeting Stat3 in cancer therapy (Jing & Tweardy *Anti-Cancer Drugs* in press (2005)).

ABSTRACT: Stat3 is constitutively activated in many human cancers where it functions as a critical mediator of oncogenic signaling through transcriptional activation of genes encoding apoptosis inhibitors (e.g., Bcl-x_L, Mcl-1 and survivin), cell-cycle regulators (e.g., cyclin D1 and c-Myc) and inducers of angiogenesis (e.g., VEGF). This article reviews several approaches that have been pursued for targeting Stat3 in cancer therapy including antisense strategies, tyrosine

kinase inhibition, decoy phosphopeptides, decoy duplex oligonucleotides and G-quartet oligodeoxynucleotides (GQ-ODN). The GQ-ODN strategy is reviewed in somewhat greater detail than the others because it includes a novel system that effectively delivers drug into cells and tissues, addresses successfully the issue of specificity of targeting Stat3 vs. Stat1 and has demonstrated efficacy *in vivo*.

Conclusion 3: This review article was summarized all agents designed to target Stat3 activity for cancer therapy. We demonstrated that (1) GQ-ODN specifically inhibits Stat3 DNA binding activity and induces apoptosis in many cancer cells. (2) A novel delivery system for GQ-ODN has been developed to increase the drug activity in cells and *in vivo*. (3) GQ-ODN T40214 and T40231 significantly suppress tumor growth and greatly increase the survival time of nude mouse xenografts with tumors where Stat3 is activated, such as prostate, breast, and SCCHN tumors. Comparing with other JAK/STAT inhibitors, GQ-ODN as a Stat3 inhibitor has advantages in selectively targeting Stat3 and strong drug effectiveness *in vivo*.

III. Reportable outcomes.

Publications:

1. Jing N, Li Y, Xiong W, Sha W, Jing L, Tweardy DJ. G-quartet oligonucleotides: a new class of signal transducer and activator of transcription 3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. *Cancer Research* 64:6003-6009 (2004).
2. Jing N, Sha W, Li Y, Xiong W, Tweardy DJ. Rational drug design of G-quartet DNA as anti-cancer agents. *Current Pharmaceutical Design* (in press) (2005).
3. Jing N, Tweardy DJ. Targeting Stat3 in cancer therapy. *Anti-Cancer Drugs* (in press) (2005)

Presentations:

1. Jing N, Li, Y., Xiong, W., Sha, W. Jing, L. Tweardy, D. J. "G-quartet oligonucleotides: a new class of Stat3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis" 95th AACR conference, 2004.
2. Jing presentation in Breast Center, Baylor College of Medicine, January 4, 2005.
3. Jing presentation in Nanjing Medical University, Najing China, Mar 16, 2005.
4. Jing presentation in Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, March 23, 2005.

IV. Conclusion of the researches from 4/1/04 to 3/31/05.

The progresses of the research work achieved in PI's lab focused in few aspects. (i) We have established a structure-activity relationship between GQ-ODN and target molecule. With the establishment, it is possible to design or screen for a more bioactive Stat3 inhibitor. (ii) We demonstrated that GQ-ODN specifically targets DNA-binding activity of Stat3 but not Stat1. Specifically targeting Stat3 among other STAT protein members is a highly desirable since other STAT proteins notably Stat1, which is activated along with Stat3 in many tumor systems (9). However, Stat3 and Stat1 use quite different strategies for controlling cell growth and apoptosis. Stat3 is an oncogene and Stat1 has pro-apoptosis function, resulting in very different effects (10, 11). (iii) We successfully delivered GQ-ODN into tumors of nude mice xenograft model and demonstrated that the GQ-ODN T40214 and T40231 as novel anti-cancer agents significaly

suppress the growth of prostate and breast tumors *in vivo*. Also we also demonstrated that blocking growth of prostate and breast tumors was based upon increase in apoptosis of cancer cells through inhibition of Stat3 activity. These results make a significant progress for developing a novel anti-cancer agent through targeting Stat3 in prostate cancer therapy.

V. References.

1. Bowman T, Garcia R, Turkson J, Jove, R. STATs in oncogenesis. *Oncogene* 2000; 19:2474-2488.
2. Bromber JF. Activation of STAT proteins and growth control. *BioEssay* 23:161-169, 2001.
3. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumor provides novel molecular targets for therapeutic intervention. *Clin. Cancer Res.* 8:945-954, 2002.
4. Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T, Falcone R, Fairclough R, Cantor A, Muro-Cacho C, Livingston S, Karras J, Pow-Sang J, Jove R. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. *Cancer Research* 62: 6659-66, 2002.
5. Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activatior of transcription 3 and phospho-state 3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. *Clinical Cancer Res.* 9: 594-600, 2003.
6. Nagpal JK, Mishra R, Das BR. Activation of Stat3 as one of early events in tobacco chewing-mediated oral carcinogenesis. *Amecrican Cancer Sociaty* 94:2393-4000, 2002.
7. Hsiao J-R, Jin Y-T, Tsai S-T, Shiau A-L, Wu C-L, Su, W-C. Constitutive activation of Stat3 and Stat5 is present in majority of nasopharyngeal carcinoma and correlates with better prognosis. *Britich J. Cancer* 2003; 89: 344-9.
8. Jing N, Li Y, Xu X, Li P, Feng L, Tweardy D. Targeting Stat3 with G-quartet oligonucleotides in human cancer cells. *DNA & Cell Biology* 2003; 22: 685-96.
9. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumor provides novel molecular targets for therapeutic intervention. *Clin. Cancer Res.* 8:945-954, 2002.
10. Bromberg JF, Horvath CM et al. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc Natl Acad Sci U S A* 93:7673-7678, 1996.
11. Xu X, Fu XY et al. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res* 58:2832-2837,1998.

VI. Appendices (attached).

1. Jing N, Li Y, Xiong W, Sha W, Jing L, Tweardy DJ. G-quartet oligonucleotides: a new class of signal transducer and activator of transcription 3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. *Cancer Research* 64:6003-6009 (2004).
2. Jing N, Sha W, Li Y, Xiong W, Tweardy DJ. Rational drug design of G-quartet DNA as anti-cancer agents. *Current Pharmaceutical Design* (in press) (2005).

[CANCER RESEARCH 64, 6603–6609, September 15, 2004]

G-Quartet Oligonucleotides: A New Class of Signal Transducer and Activator of Transcription 3 Inhibitors That Suppresses Growth of Prostate and Breast Tumors through Induction of Apoptosis

Naijie Jing,[1] Yidong Li,[1] Weijun Xiong,[1] Wei Sha,[1] Ling Jing,[2] and David J. Tweardy[1]

[1]*Department of Medicine, Section of Infectious Diseases, Baylor College of Medicine; and University of Texas Health Science Center, School of Public Health, Houston, Texas*

ABSTRACT

***Stat3* is a signaling molecular and oncogene activated frequently in many human malignancies including the majority of prostate, breast, and head and neck cancers; yet, no current chemotherapeutic approach has been implemented clinically that specifically targets Stat3. We recently developed G-rich oligodeoxynucleotides, which form intramolecular G-quartet structures (GQ-ODN), as a new class of Stat3 inhibitor. GQ-ODN targeted Stat3 protein directly inhibiting its ability to bind DNA. When delivered into cells using polyethyleneimine as vehicle, GQ-ODN blocked ligand-induced Stat3 activation and Stat3-mediated transcription of anti-apoptotic genes. To establish the effectiveness of GQ-ODN as a potential new chemotherapeutic agent, we systemically administered GQ-ODN (T40214 or T40231) plus polyethyleneimine or polyethyleneimine alone (placebo) by tail-vein injection into nude mice with prostate and breast tumor xenografts. Whereas the mean volume of breast tumor xenografts in placebo-treated mice increased >7-fold over 18 days, xenografts in the GQ-ODN-treated mice remained unchanged. Similarly, whereas the mean volume of prostate tumor xenografts in placebo-treated mice increased 9-fold over 10 days, xenografts in GQ-ODN-treated mice increased by only 2-fold. Biochemical examination of tumors from GQ-ODN-treated mice demonstrated a significant reduction in Stat3 activation, levels of the antiapoptotic proteins Bcl-2 and Bcl-x_L, and an 8-fold increase in the number of apoptotic cells compared with the tumors of placebo-treated mice. Thus, GQ-ODN targeting Stat3 induces tumor cell apoptosis when delivered into tumor xenografts and represents a novel class of chemotherapeutic agents that holds promise for the systemic treatment of many forms of metastatic cancer.**

INTRODUCTION

Signal transducer and activator of transcription (STAT) proteins were originally discovered as latent cytoplasmic transcription factors (1). STAT proteins, including Stat1, 2, 3, 4, 5a, 5b, and 6 link to a variety of cellular and biological processes including proliferation, differentiation, apoptosis, host defense, and transformation (2–6). STAT proteins are localized within the cytoplasm of resting cells and become activated by tyrosine phosphorylation at their COOH-terminal end (Y705) after recruitment to activated receptor complexes. Tyrosine phosphorylation induces formation of dimers, which translocate to the nucleus, where they bind to DNA-response elements in the promoters of target genes and activate transcription (7).

Stat3, previously termed acute phase response factor (APRF; refs. 2, 8–10), is activated within cells by binding to the cell surface of >40 ligands; it also is constitutively activated in many human cancers (11–13) including 82% of prostate cancers (14), 69% of breast cancers (15), 82 to 100% of squamous cell carcinoma of head and neck (16), and 71% of nasopharyngeal carcinoma (17). Activated Stat3 up-regulates the expressions of antiapoptosis proteins, such as Bcl-x_L and Mcl-1, thereby decreasing spontaneous apoptosis in cancer cells (18, 19). Targeting of Stat3 directly with agents, such as antisense oligonucleotides (20–22), a decoy oligonucleotide (23), or indirectly with Janus-activated kinase inhibitors, AG490 and JSI-124 (24, 25), demonstrated that Stat3 activation contributes to tumor cell growth and resistances to apoptosis, providing the strongest rationale for therapeutic approaches aimed at reducing levels or activity of Stat3 in cancers where it is activated.

Stat3, similar to all of the STAT proteins, is composed of several domains: a tetramerization domain, a coil-coil domain, a DNA-binding domain, a linker domain, an SH2 domain, a critical tyrosine reside (Y705), and a COOH-terminal transactivation domain. Resolution of the crystal structure of Stat3 revealed the structural basis for DNA-binding and dimer formation in sufficient detail to provide primary targets for novel drug development (26). One class of drug in early development as a novel agent targeting Stat3 is G-rich oligodeoxynucleotides (ODNs) that form G-quartet structures intracellularly (27).

G-rich DNA and RNA have the ability to form inter- and intramolecular four-stranded structures, referred to as G-quartets (28, 29). G-quartets arise from the association of four G-bases into a cyclic Hoogsteen H-bonding arrangement, and each G-base makes two H-bonds with its neighbor G-base (N1 to O6 and N2 to N7). G-quartets stack on top of each other to give rise to tetrad-helical structures. The stability of G-quartet structures depends on several factors: the presence of the monovalent cations, the concentration of the G-rich oligonucleotides present, and the sequence of the G-rich oligonucleotides under study. Potassium with the optimal size to interact within a G-octamer greatly promotes the formation of G-quartet structures and increases their stability. G-quartet oligodeoxynucleotides (GQ-ODNs) have been suggested to play a critical role in several biological processes including modulation of telomere activity (30), inhibition of human thrombin (31), HIV infection (32), HIV-1 integrase activity (33–35), human nuclear topoisomerase 1 activity (36), and DNA replication *in vitro* (37). On the basis of the structure and mechanism of Stat3 activation, G-quartet–forming oligonucleotides were developed recently to block Stat3 activity within cancer cells (27). GQ-ODNs directly target Stat3 protein and inhibit their ability to bind DNA thereby decreasing transcriptional activation of genes important for apoptosis resistance, such as *Bcl-x* and *Mcl-1*.

In the present report, we demonstrated that i.v. administration of GQ-ODNs dramatically reduces the growth in nude mice of xenografts of prostate and breast cancer cells in which Stat3 is constitutively activated. GQ-ODNs inhibited tumor cell growth by markedly enhancing tumor cell apoptosis and representing a promising agent for treatment of metastatic tumors in which Stat3 is constitutively activated.

MATERIALS AND METHODS

Materials. All of the G-rich ODNs including 5′-fluorescein-labeled ODN were synthesized by Midland Certified Reagent Company (Midland, TX) and used without additional chemical modifications. The human cell lines HepG2,

Received 12/24/03; revised 7/7/04; accepted 7/13/04.

Grant support: Department of Defense Award PC020407 and NIH Grants GM60153 and CA86430.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Naijie Jing, Department of Medicine, Section of Infectious Diseases, Baylor College of Medicine, Houston, TX. Phone: 713-798-3685; Fax: 713-798-8948; E-mail: njing@bcm.tmc.edu.

PC-3, and MDA-MB-468 were obtained from the American Type Culture Collection (Rockville, MD). Polyethylenimine (~25 kDa polymer, Sigma-Aldrich, Inc., St. Louis, MO) was generously provided by Dr. Charles Densmore (Baylor College of Medicine). Interleukin (IL) 6 and antibodies against Stat3, Stat1, Bcl-x_L, and Bcl-2 were purchased from Santa Cruz Biotechnology Biotechnology (Santa Cruz, CA). Antibody against caspase 3 was obtained from Cell Signaling Technology (Beverly, MA).

Assay of Inhibition by GQ-ODNs of Stat3 DNA-Binding Activity in Cancer Cells. GQ-ODN plus polyethyleneimine at a weight ratio of polyethyleneimine/ODN of 2:1 was added to cells (5–7 × 10^5). After incubation for 3 hours, the cells were washed three times with fresh medium without polyethyleneimine/ODN and the incubation continued. After 6 to 72 hours, cells were incubated without or with IL-6 (25 ng/mL) at 37°C for 20 minutes before extraction and analysis by electrophoretic mobility shift assay. The cell extraction and electrophoretic mobility shift assay were performed as described previously (27). In some experiments, cells were scraped and harvested for isolation of nuclear proteins as described previously (38). Briefly, 50 μL of ice-cold Buffer A [10 mmol/L Tris-HCL (pH 9.), 2 mmol/L $MgCl_2$, 5 mmol/L KCl, 10% glycerol, 1 mmol/L EDTA, and 1 mmol/L DDT] plus 1% NP40 was added and the cells allowed to swell on ice for 15 minutes, then vortexed vigorously. The lysate was centrifuged at 700 × *g* for 5 minutes at 4°C and the resulting nuclear pallet resuspended in 50 μL of cold buffer (20 mmol/L HEPES, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L DDT). Nuclear proteins were extracted by vigorous shaking for 30 minutes at 4°C and cell debris pelleted by centrifugation at 18,000 × *g* for 5 minutes at 4°C. Protein concentrations of whole-cell and nuclear extracts were measured by Bradford assay.

Statistical Docking Calculations. The structures of the designed G-quartet ODNs, such as T40214 and its analogues, were built up through modification of the nuclear magnetic resonance structure of GQ-ODN T30923 (39) and optimized under AMBER force field by INSIGHTII/DISCOVER. The optimization of the molecular structures proceeded as follows: (1) 100 steps of conjugate gradient energy minimization, (2) 1,000 steps of restrained MD equilibration with a time step of 0.33 fs at 1,000 K, (3) 1,000 steps of restrained MD equilibration with a time step of 0.1 fs at 300 K, and (4) 1,000 steps of conjugate gradient energy minimization. The intramolecular H-bonds of G-quartets were used as constraints for the molecular optimization.

We docked each GQ-ODN 1,000 times onto the available structure of the dimer of Stat3 SH2 (26) using the GRAMM docking program without placing any restrictions on binding sites. This program uses a geometry-based algorithm for predicting the structures of complexes between molecules of known structure. It can provide quantitative data related to the quality of the contact between the molecules. The intermolecular energy calculation relies on the well-established correlation and Fourier transformation techniques used in the field of pattern recognition. The distribution of H-bond formation between each Stat3/GQ-ODN complex was calculated and analyzed.

***In vivo* Delivery of Fluorescently Labeled GQ-ODN.** Fluorescent-labeled T40214 plus polyethyleneimine (each 2.5 mg/kg) was injected into the tail vein of male and female mice weighing ~20 g. Twenty-four hours after the infusion, the mice were sacrificed and tissue harvested and frozen. Frozen tissues were sectioned using cryostat microtome sections, lightly fixed, and viewed microscopically.

Tumor Xenograft Models. Athymic nude mice (Balb/*nu/nu*, 4 weeks old and weighing ~20 g obtained from Charles River Labs) were injected s.c. into the right (or left) flank with one million cancer cells (MDA-MD-468 or PC-3) in 200 μL of PBS. After tumors were established at 7 to 14 days postinjection, 16 nude mice with breast or prostate tumors were randomly assigned into three groups. Mice in group 1 (n = 7) served as placebo and received only polyethyleneimine (2.5 mg/kg), whereas mice in groups 2 and 3 (n = 7) received T40214 (5.0 mg/kg) plus polyethyleneimine (2.5 mg/kg) and T402314 (5.0 mg/kg) plus polyethyleneimine (2.5 mg/kg), respectively. Treatments, administered by tail-vein injection, and sizing of tumors occurred every 2 days. The unpaired two-sample t test, $\{t = (X_1 - X_2)/[S_p^2(1/n_1+1/n_2)]^{1/2}\}$, was used to determine differences in tumor sizes between the placebo and the drug-treated groups.

Immunoblotting. Tumor xenografts were harvested at the end of treatment period, cut into small pieces, homogenized on ice for 2 minutes, subjected to one round of freeze-thawing, and centrifuged (12,000 rpm for 2 min at 4°C). The supernatants were harvested, assayed for protein concentration by Bradford assay, and immunoblotted as described elsewhere (20).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End-Labeling Assay. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed as described by the manufacturer (Roche Applied Science, Indianapolis, IN). Briefly, prostate (or breast) tumors were harvested from mice within 24 hours of their last treatment. The paraffin-embedded tissues were pretreated using Xylene and EtOH, washed with PBS, and incubated with proteinase K working solution. TUNEL reaction mixture was prepared by adding 50 μL of enzyme solution into 450 μL of the labeling solution. TUNEL reaction mixture (50 μL) was placed on slides followed by 50 μL of 3,3′-diaminobenzidine substrate. Slides were incubated for 5 minute at 24°C, rinsed three times with PBS, dehydrated with xylene, and mounted under a glass coverslip.

RESULTS

Inhibition of Stat3 Activation by G-Quartet ODNs in Cancer Cells. Stat3 is expressed and constitutively activated in the prostate cancer cell line PC-3 and the breast cancer cell line MDA-MB-468 (14, 24). The GQ-ODN T40214 and a panel of related G-rich ODNs capable of forming G-quartets were mixed with polyethyleneimine, and each was examined for the ability to inhibit Stat3 activation in these cell lines (Fig. 1, *A–C*; Table 1). Each of the G-rich ODN demonstrated the ability to inhibit Stat3 DNA-binding activity when the concentration of ODN was increased from 7 to 285 μmol/L. T40214 and T40231 are the most potent inhibitors of Stat3 activation. Nonspecific ODNs incapable of forming the G-quartet structure demonstrated no activity against Stat3.

Structure–Activity Relationship between Stat3 and G-Quartet Inhibitors. T40214 was determined previously to form an intramolecular G-quartet structure composed of two G-quartets in the center and two G-C-G-C loop domains on the top and bottom with ~15Å

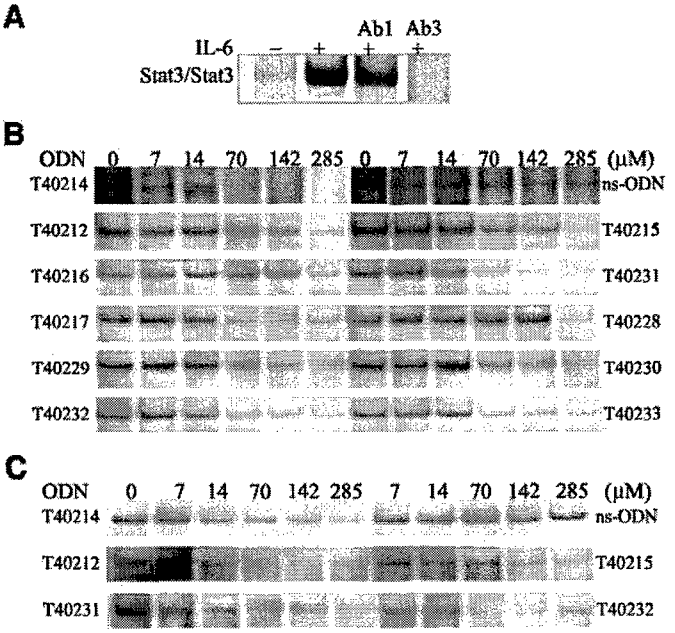


Fig. 1. Inhibition of Stat3 activation in prostate and breast cancer cells by GQ-ODN. *A*, Stat3 is activated constitutively and by IL-6 in PC-3 cells. Electrophoretic mobility shift assay was performed using extracts of PC-3 cells without (−) and with (+) stimulation with IL-6 (25 ng/ml); extracts were incubated without or with antibodies against Stat1 (*Ab1*) and Stat3 (*Ab3*). Electrophoretic mobility shift assay was performed using extracts of IL-6-stimulated PC-3 (*B*) and MDA-MB-468 cells (*C*) pretreated with each panel of ODN (see Table 1 for sequences). Cells were preincubated with the indicated ODN plus polyethyleneimine at the indicated concentration for 3 hours, washed, and incubated in fresh medium for 24 hours, and then stimulated with IL-6 (25 ng/ml) before extraction.

Table 1 *IC_{25}, IC_{50}, and IC_{75} of ODN for inhibition of IL-6–stimulated Stat3 activation in PC-3 cells and percentage of total H-bond formed between ODN and the region of Stat3 SH2 located between amino acid residues 638 and 650*

Compound	5′-sequence	IC_{25} (μM)	IC_{50} (μM)	IC_{75} (μM)	% H-bounds formed within Stat3 (aa 638–650)
Ns-ODN	TGCCGGATCCAAGAGCTACCA				
T40214	GGGCGGGCGGGCGGGC	2.5	5.0	24	35
T40212	GGGCGGGTGGGCGGGT	17	35	142	30
T40215	$(GGGGT)_4$	12	49	163	28
T40216	$(GGGGGT)_4$	30	202		
T40217	GGGGTGGGTGGGTGGGTT	35	64		27
T40228	GCGGGTGGGTGGGTGGGTCG	139	192	244	
T40229	TAGGGTGGGTGGGTGGGTAT	9	41	248	29
T40230	GTGGGTGGGTGGGTGGGTTG	11	43		28
T40231	GGTGGGTGGGTGGG	4.6	10	49	33
T40232	GGCGGGCGGGCGGG	10	26	62	32
T40233	GGGCGGGTGGGCGG	7.6	34	67	29

width and 15Å length (39, 40). T40214 and other GQ-ODNs destabilize Stat3 dimers by inserting between their SH2 domains. We proposed that GQ-ODNs interact in the region of the SH2 domain from amino acid residues 638 to 650 (ref. 27; Fig. 2*A*). To establish a structure–activity relationship between Stat3 and G-quartet inhibitors, we generated the G-quartet structure predicted to be formed by each G-rich ODN (Table 1) and randomly docked each of them onto the known structure of the dimer of Stat3 SH2 domains (26) 1,000 times. Analysis of the histogram of H-bonds formed at each residue within the Stat3 SH2 domain demonstrated that T40214 interacts with SH2 predominantly in the region from residues 638 to 650, as expected, with 35% of total H-bonds formed located in this region (Fig. 2*B*). The percentages of H-bonds formed within the region from residues 638 to 650 of Stat3 for other GQ-ODNs were listed in Table 1. Composite analysis of all of the histograms revealed that there is an inverse linear correlation between the percentage of GQ-ODN H-binding within this region of the Stat3 SH2 domain and the IC_{50} of inhibition of Stat3 DNA binding activity (Fig. 2*C*). Thus, these findings indicate that the higher the percentage of GQ-ODN H-bond formation within this region of Stat3 SH2, the greater its ability to inhibit Stat3 activation within cells.

GQ-ODN–Mediated Inhibition of Stat3 within Cells Is Selective for Stat3, Requires Polyethyleneimine, and Is Accompanied by Decreased Levels of Intranuclear Stat3 Phosphorylated on Y705 (Stat3-pY705). We demonstrated previously that GQ-ODN had 4-fold greater activity against Stat3 than Stat1 *in vitro* (27). To determine whether this selectively persisted or increased upon intracellular delivery, we preincubated HepG2 cells, in which both Stat3 and Stat1 are activated by IL-6, with T40214 (0–142 μmmol/L) for 72 hours before stimulation with IL-6. Preincubation of HepG2 cells with T40214/polyethyleneimine complex at 70 μmmol/L (or higher) nearly completely inhibited IL-6-mediated activation of Stat3, whereas activation of Stat1 was only mildly affected (Fig. 3, *A* and *C*) confirming the selectivity of GQ-ODN for Stat3 within cells. No inhibition of Stat3 DNA binding activity was observed when HepG2 cells were preincubated with T40214 in the absence of polyethyleneimine (Fig. 3*B*) confirming previous findings by us (40) that a vehicle is required for delivery of GQ-ODN into cells.

To determine whether GQ-ODNs also interfere with Stat3 activation and translocation into the nucleus, we examined levels of Stat3-pY705 within the nucleus of cells stimulated with IL-6 after GQ-ODN pretreatment. Levels of Stat3-pY705 were reduced from 50 to 70% in nuclei when the concentration of T40214 was increased from 3.5 to 142 μmmol/L (Fig. 4, lanes 4 to 7). polyethyleneimine alone as vehicle has no effect on Stat3 activation (Fig. 4, lane 3).

Delivery of G-Quartet ODN into Tumor Xenografts Results in Inhibition of Growth through Induction of Apoptosis. To determine whether GQ-ODNs can be administered to mice and delivered

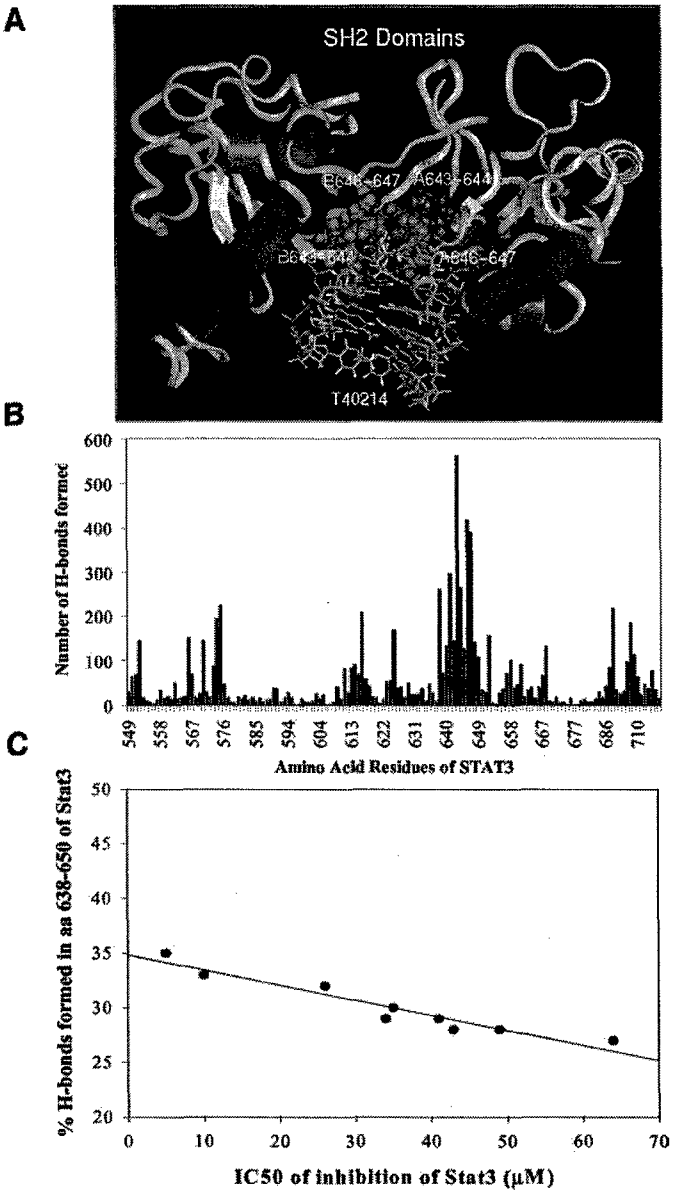


Fig. 2. Structure-activity relationship of GQ-ODNs. *A*, model of T40214 bound to dimers of Stat3 SH2 showing T40214 (*blue wire model*) interacting with residues Q643, Q644, N646, and N647 of Stat3 dimer (*green space-filling model*). *B*, histogram of the distribution of all H-bonds formed between T40214 and the SH2 domain of Stat3 in 1,000 computer-simulated dockings. *C*, plot demonstrating the relationship between the percentage of GQ-ODN H-bonding localized from residues 638 to 650 and its IC_{50} against Stat3 (Table 1; $r^2 = 0.91$; $P < 0.001$).

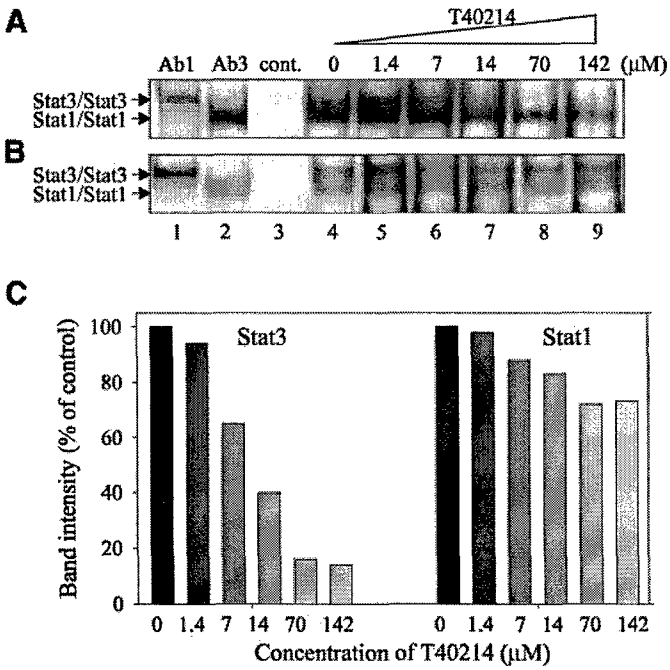


Fig. 3. GQ-ODN selectively targets Stat3 within cells, and its delivery requires polyethyleneimine. Electrophoretic mobility shift assay of extracts of HepG2 cells stimulated with IL-6 (25 ng/ml) 72 hours after preincubation of cells with T40214 at the indicated concentrations complexed with (*A*) or without (*B*) polyethyleneimine. Extracts were incubated with antibodies against Stat1 (*Ab1*; *Lane 1*) or Stat3 (*Ab3*; *Lane 2*) to confirm the composition of the homodimer bands. *C*, Intensity of the homodimer bands in *lanes 4* through *9* of T40214 with polyethyleneimine (*A*) were measured by densitometry and plotted as a percentage of the band in the untreated lane (0 μmol/L; *lane 4*).

into tumor xenografts, we injected fluorescein-labeled T40214 plus polyethyleneimine (each at 2.5 mg/kg) into the tail veins of nude mice with established tumor xenografts. Microscopic examination of xenografts 24 hours after injection demonstrated diffuse fluorescent staining within tumor cells (Fig. 5*A*) indicating that GQ-ODN enters and accumulates within tumor cells.

The two most active GQ-ODNs, T40214 and T40231 (Fig. 5*B*), were selected from among the designed GQ-ODNs to assess if GQ-ODN uptake is accompanied by inhibition of tumor growth. Nude mice were injected s.c. with MDA-MB-468 or PC3, both of which demonstrate constitutive Stat3 activity, and i.v. treated every other day after tumors were established (Fig. 5, *C* and *D*). The drug treatment groups received T40214 or T40231 (5.0 mg/kg) plus polyethyleneimine (2.5 mg/kg), and the placebo groups each received polyethyleneimine (2.5 mg/kg) alone by tail-vein injection. The mean size of the breast tumor xenografts of placebo-treated mice increased from 7- to 12-fold, whereas the mean sizes of both T40214 and T40231-treated mice remained unchanged (Fig. 5*C*). The growth rate of placebo-treated breast tumors ranged from 11% to 15%/day, and those of T40214 and T40231-treated breast tumors were −0.4%/day ($P = 0.001$) and 0.6%/day ($P = 0.001$), respectively. The mean size of the prostate tumor xenografts of placebo-treated mice increased 9- to 10-fold, whereas the mean sizes of T40214-treated and T40231-treated mice increased only 2.2- and 2.6-fold, respectively (Fig. 5*D*). The growth rate of placebo-treated prostate tumors ranged from 21% to 23%/day, whereas the growth of T40214- and T40231-treated prostate tumors was 9.1%/day ($P = 0.001$) and 8.8%/day ($P = 0.001$), respectively.

To gain insight into the mechanism of inhibition of tumor growth by GQ-ODN, we harvested the prostate tumor xenografts from placebo-treated and drug-treated mice after five treatments and extracted proteins to assess for levels of Stat3-pY705, Bcl-x_L, Bcl-2, and activated caspase 3 protein. Levels of Stat3-pY705, Bcl-x_L, and Bcl-2 were decreased by 9-, 4.3-, and 10-fold, respectively, in the tumors from drug-treated animals compared with tumors from placebo-treated mice. These changes were accompanied by a 3-fold increase in caspase 3 cleavage products in the tumors from drug-treated animals compared with tumors from placebo-treated mice (Fig. 6*A*). We have harvested the 4 tumor samples from placebo-treated mice and 3 tumor samples from drug-treated mice and then performed TUNEL assay on the samples. TUNEL staining was performed on 4 tumor samples from placebo-treated mice and 3 tumor samples from drug-treated mice to assess if GQ-ODN treatment increased tumor cell apoptosis. The percentage of apoptotic cells was increased nearly 8-fold in the tumors of drug-treated mice (83.6 ± 1.0%) compared with the tumors of placebo-treated mice (11.2 ± 10.1%; $P < 0.001$; Fig. 6*B*).

DISCUSSION

Mounting evidence from cell culture, whole animals, and patient samples indicates that Stat3 is a critical mediator of oncogenic signaling that is activated in many human malignancies (17). This evidence provides a strong rationale for developing agents that target Stat3 for treatment of cancers in which constitutive Stat3 activation plays a critical role. Currently, no chemotherapeutic approach has been implemented that targets Stat3. We recently developed G-rich oligodeoxynucleotides, which form intramolecular G-quartet structures (GQ-ODNs), as a new class of Stat3 inhibitor; GQ-ODNs were shown to target Stat3 dimers predominantly in the regions of their SH2 domains resulting in their destabilization and reduced ability to bind DNA *in vitro* (27). In studies reported here, we demonstrate that when delivered into cells using polyethyleneimine as vehicle, GQ-ODNs blocked ligand-induced Stat3 activation. We determined that the region within the SH2 dimer domains predominantly bound by

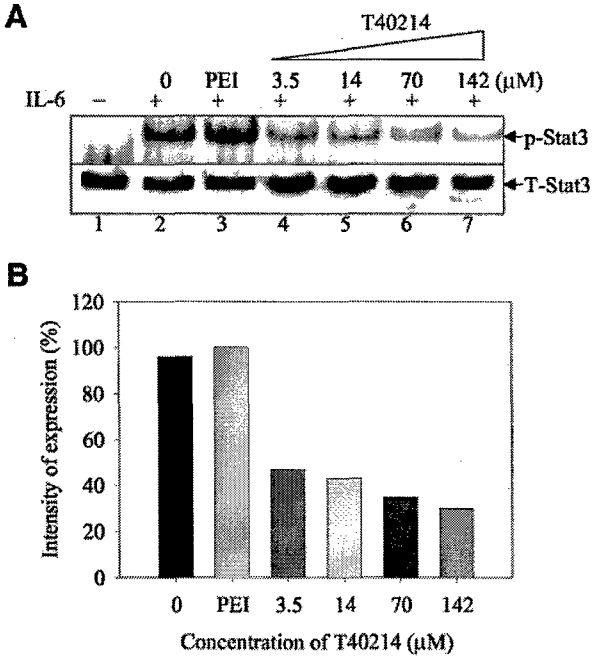
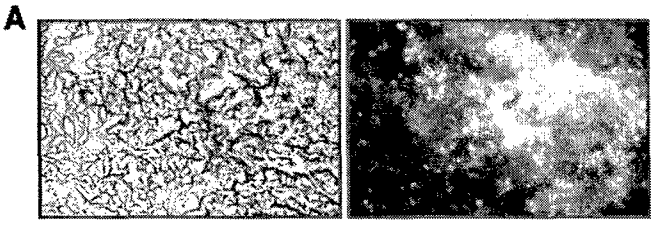
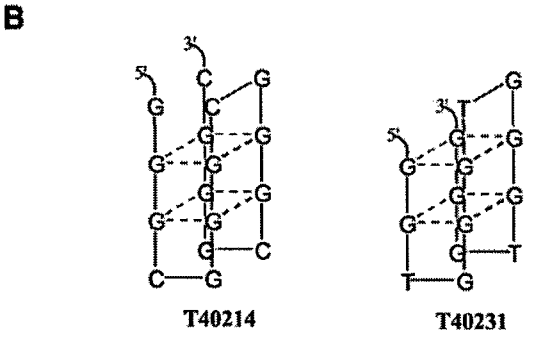
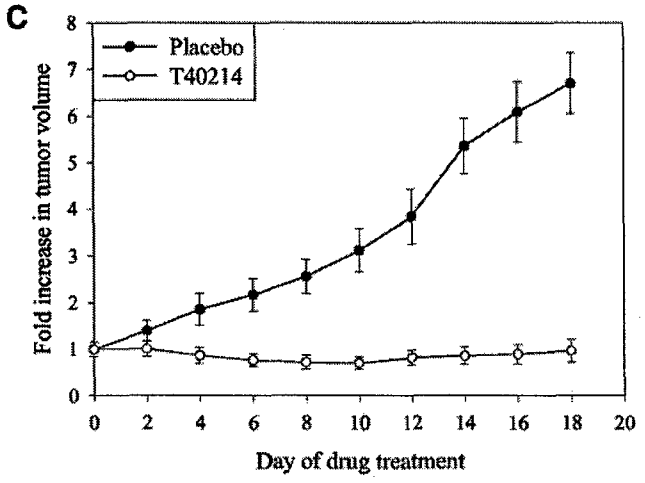
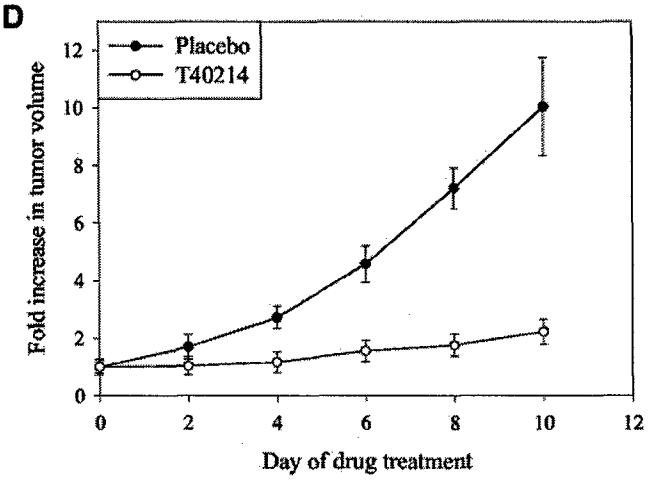


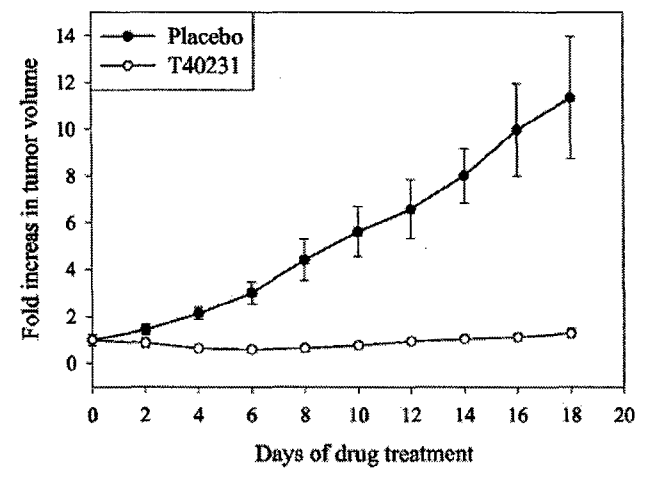
Fig. 4. GQ-ODN inhibit IL-6-mediated increase in intranuclear phosphorylated Stat3. *A*, immunoblot of nuclear extracts of HepG2 cells preincubated with media (*0*), polyethyleneimine alone (*PEI*), or T40214 at the indicated concentrations for 3 hours. Cells were then washed and incubated in medium alone for 24 hours before stimulation with IL-6 (25 ng/mL); nuclear extracts were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody (pY705; *top*) or Stat3 monoclonal antibody (T-Stat3, *bottom*). *B*, The p-Stat3 bands were quantitated by densitometry and plotted as the percentage of the polyethyleneimine-pretreated lane (*lane 3*).











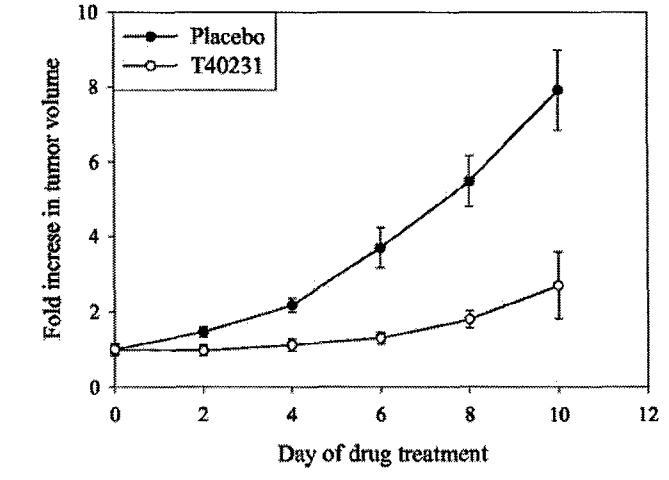


Fig. 5. GQ-ODN is successfully delivered into tumor xenografts and inhibits their growth. *A*, light microscopic (*left*) and fluorescent microscopic (*right*) photomicrographs (each ×400 magnification) of a tumor xenograft harvested from a nude mouse 24 hours after tail-vein injection with fluorescein-labeled T40214/polyethyleneimine complex (each at 2.5 mg/kg), showing the uptake of T40214 within tumor cells. *B*, the models of T40214 and T40231 structures. Breast (*C*) and prostate (*D*) cancer cells xenograft volumes were measured at the start of therapy and every other day in mice treated with T40214/polyethyleneimine complex (○) or with polyethyleneimine alone (●; *top*) or with T40231/polyethyleneimine complex or with polyethyleneimine alone (*bottom*). Data plotted are fold increase in tumor volume of each group; *bars*, ±SE.

GQ-ODN was located between residues 638 and 650, a region critically involved in Stat3 dimerization. Importantly, we established a linear structure-activity relationship that directly correlates the ability of a GQ-ODN to bind to this region of Stat3 SH2 and its ability to inhibit Stat3 activation within cells, which is an important step toward optimizing the design GQ-ODN inhibitors of Stat3. Furthermore, intravenous administration of GQ-ODNs plus polyethyleneimine blocked the growth of breast and prostate tumor xenografts in nude mice. This effect was accompanied by marked reductions in levels of Stat3 activation and Bcl-2 and Bcl-x_L protein and a striking increase

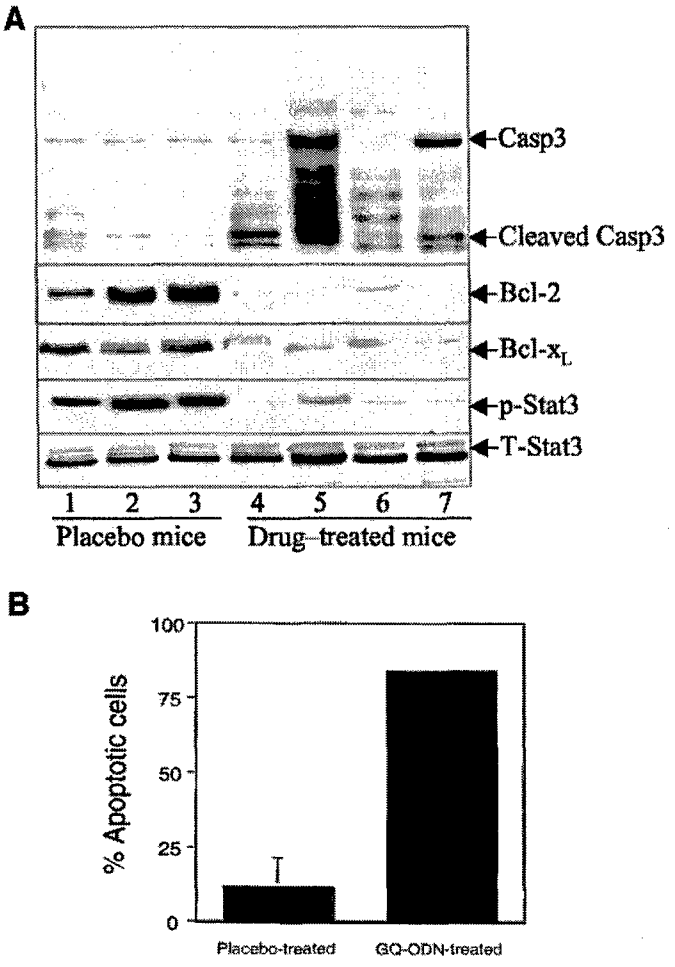


Fig. 6. Effect of G-quartet ODN T40214 on apoptosis and levels of apoptosis-related proteins in tumor xenografts. *A*, Proteins were extracted from tumor xenografts obtained from 3 placebo-treated mice (*lanes 1* to *3*) and from 4 drug-treated mice (*lanes 4* to *7*), separated by SDS-PAGE, and immunoblotted with the antibodies indicated. Each lane was loaded an equal amount of the total *Stat3 (T-Stat3)* protein as a control. *B*, percentage of apoptotic cells within prostate tumor xenografts assessed by TUNEL staining. Two hundred cells within TUNEL stained sections of prostate tumor xenografts removed on day 10 from placebo-treated mice (n = 4) and GQ-ODN-treated mice (n = 3) were examined at ×400 magnification. Data presented are the mean; *bars*, ±SD; $P < 0.001$.

in tumor cell apoptosis. Thus, GQ-ODNs, which target Stat3 and induce apoptosis within tumor xenografts after intravenous administration, represent a novel cancer chemotherapeutic approach that holds promise for the systemic treatment of many forms of metastatic cancer.

Our previous studies showed that the G-quartet structure of G-rich ODN is essential for the inhibition of Stat3 DNA-binding activity *in vitro* (27). The G-quartet structure of T40214 closely resembles a perfect cylinder 15 Å in width and length. This conformation increases the thermal stability of the structures, reduces the capacity of ODN to form molecular aggregates, and increases the probability that each GQ-ODN will target Stat3 homodimer in cells. The finding that T40214-mediated inhibition of ligand-induced Stat3 activation within cells persisted for 72 hours after GQ-ODN exposure can be attributed, in part, to this compact configuration, which contributes to its thermal stability and resistance to nuclease digestion. The intramolecular G-quartet structure prevents single-strand endonucleases from accessing their cleavage sites (41).

Effective delivery of GQ-ODNs into cancer cells is a key factor for success of inhibitors that target oncogenic signaling intermediates. On the basis of the property of potassium-dependent formation of G-quartet structure, a novel intracellular delivery system has been developed for GQ-ODNs (40). Using the novel delivery system, GQ-ODNs were delivered efficiently into the cytoplasm and nucleus of cells. The results reported here showed that GQ-ODNs penetrated poorly into cells without vehicle as evidenced by low drug activity, whereas use of an effective intracellular delivery system such as polyethyleneimine increased the drug activity of GQ-ODNs in cancer cells (Fig. 3, *A* and *B*). When G-rich ODNs form intramolecular G-quartet structures within the cytoplasm, they are able to diffuse freely through pores into the nucleus, bind to Stat3, and block the transcription of Stat3-regulated genes (Fig. 4; ref. 27).

Because of the important role that STAT proteins play in signaling within the immune system (Stat1, 2, 4, 5A, 5B, and 6) and in negative regulation of proliferation (Stat1; ref. 42), it is highly desirable that an agent targeting Stat3 not have activity against other STAT proteins to avoid problems with immunosuppression and inadvertent stimulation of tumor cell growth. On the basis of their structures (14, 43), the ligand-receptor pairs that lead to their activation (1) and the phosphotyrosine sites that lead to their recruitment and activation (44), the STAT protein family member that most closely resembles Stat3 is Stat1. Stat3 and Stat1 share >50% amino acid sequence homology in the region of the SH2 domain (13). Therefore, to address the issue of specificity of GQ-ODN inhibition, we focused on Stat1. In previous studies, we demonstrated that the IC_{50} of T40214 for the inhibition of Stat3 DNA-binding activity *in vitro* was 4-fold less than for its inhibition of Stat1 DNA-binding activity (27). Here, we demonstrated that the specificity of T40214 for Stat3 *versus* Stat1 extends *in vivo*. The concentration of T40214 that inhibited 50% of Stat3 activation after preincubation of T40214 in cells was ~10 μmol/L (Fig. 3*C*), whereas 50% inhibition of Stat1 under the same conditions could not be achieved with concentrations of T40214 up to142 μmol/L. The basis for this selectivity was explained, in part, by the analysis of histograms, which were generated by docking the structure of T40214 onto the structures of Stat3β and Stat1 homodimers 2,000 times without setting any binding site restrictions (26). These studies demonstrated that interactions of GQ-ODN with Stat3 were concentrated within the SH2 and DNA-binding domains; in contrast, interactions of GQ-ODN with Stat1 were distributed more broadly over the whole structure of Stat1.

Intravenous administration of GQ-ODN plus polyethyleneimine was well tolerated by mice at the dose used in this study. Detailed toxicity studies of T40214 and T40231 are in progress; however, toxicity studies of GQ-ODN T30177, an analogue of T40214 that inhibits HIV-1 integrase, has been performed previously (45). T30177 did not induce genetic mutations in three assays, the *Ames/Salmonella* mutagenesis assay, the Chinese hamster ovary/hypoxanthine-guanine phosphoribosyltransferase mammalian cell mutagenesis assay, and the mouse micronucleus assay. Acute toxicity studies in mice revealed an LD_{50} for T30177 of 1.5g/kg body weight; chronic toxicity studies in mice after multiple doses did not cause delayed mortality or changes in serum chemistry, hematologic parameters, or organ histology until the dose of T30177 reached 600 mg/kg, 120 times the dose (5 mg/kg) used in our studies.

The results of *in vivo* drug testing revealed that GQ-ODN T40214 dramatically suppressed the growth of xenografts of prostate and breast cancer cells in which Stat3 is constitutively activated. GQ-ODN T40214 markedly decreased the level of phosphorylated Stat3 in tumor xenografts; this decrease was associated with decreased levels of Bcl-2 and Bcl-x_L protein. Bcl-2 and Bcl-x_L are antiapoptosis proteins that have ion channel activity, which inhibit the release from mitochondria of cytochrome C (46, 47). Reduced levels of Bcl-2 and Bcl-x_L in tumor xenografts would result in increased release of cytochrome C resulting in activation of the caspase cascade, which

includes caspase 3, leading to cell apoptosis. There was a striking increase in caspase 3 cleavage products in the tumors of T40214-treated animals that was accompanied by an increase in tumor cell apoptosis of similar magnitude. Our results indicate that GQ-ODNs such as T40214 block tumor xenograft growth by targeting Stat3 and inhibiting its phosphorylation, which blocks the transcription of the antiapoptosis proteins and triggers the apoptosis of cancer cells. Thus, GQ-ODNs represent a novel and potentially promising class of drug for treatment of metastatic tumors in which Stat3 is constitutively activated as either a single agent or part of a combination regimen.

REFERENCES

1. Darnell Jr JE. STATs and gene regulation. Science 1997;277:1630–5.
2. Zhong Z, Wen Z, Darnell Jr JE. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 1994;264:95–8.
3. Bromberg JF, Horvath CM, Wen Z, Schreiber RD, Dannell Jr JE. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. Proc Natl Acad Sci USA 1996;93:7673–8.
4. Fukada T, Hibi M, Yamanaka Y, et al. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. Immunity 1996;5:449–60.
5. Planas AM, Berruezo M, Justicia C, Barron S, Ferrer I. Stat3 is present in the developing and adult rat cerebellum and participates in the formation of transcription complexes binding DNA at the sis-inducible element. J Neurochem 1997;68:1345–51.
6. Takeda K, Noguchi K, Shi W, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci USA 1997;94:3801–4.
7. Bromberg J, Darnell JE. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 2000;19:2468–73.
8. Wegenka UM, Lutticken C, Buschmann J, et al. The interleukin-6-activated acute-phase response factor is antigenically and functionally related to members of the signal transducer and activator of transcription (STAT) family. Mol Cell Biol 1994; 14:3186–96.
9. Lutticken C, Wegenka UM, Yuan J, et al. Association of transcription factor APRF and protein kinase jak 1 with the interleukin-6 signal transducer gp130. Science 1994;263:89–92.
10. Raz R, Durbin JE, Levy DE. Acute phage response factor and additional member of the interferon-stimulated gene factor 3 family integrate diverse signals from cytokines, interferons, and growth factor. J Biol Chem 1994;269:24391–5.
11. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000; 19:2474–88.
12. Bromber, J.F. Activation of STAT proteins and growth control. BioEssay 2001;23: 161–9.
13. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumor provides novel molecular targets for therapeutic intervention. Clin Cancer Res 2002;8:945–54.
14. Mora LB, Buettner R, Seigne J, et al. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002;62:6659–66.
15. Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activatior of transcription 3 and phospho-state 3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clinical Cancer Res 2003;9:594–600.
16. Nagpal JK, Mishra R, Das BR. Activation of Stat3 as one of early events in tobacco chewing-mediated oral carcinogenesis. Amecrican Cancer Sociaty 94:2393–400.
17. Hsiao J-R, Jin Y-T, Tsai T, Shiau A-L, Wu C-L, Su W-c. Constitutive activation of Stat3 and Stat5 is present in majority of nasopharyngeal carcinoma and correlates with better prognosis. Britich J Cancer 2003;89:344–9.
18. Boise LH, Gonzalez-Garcia M, Postema CE, et al. Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993;74:597–608.
19. Zhou P, Qian L, Kozopas KM, Craig RW. Mcl-1, a bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 1997;89:630–43.
20. Grandis JR, Drenning SD, Chakraborty A, et al. "Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth in vitro." J Clin Invest 1998;102:1385–92.
21. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. Proc Natl Acad Sci USA 2000;97:4227–32.
22. Barton BE, Karras JG, Murphy TF, Barton A, Huang HF-S. signal transducer and activatior of transcription 3 (STAT3) activation in prostate cancer: direct Stat3 inhibition induces apoptosis in prostate cancer lines. Mol Cancer Ther 2004;3:11–20.
23. Leong PL, Andrews GA, Johnson DE, et al. targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci USA 2003;97:4138–43.
24. Burdelya L, Catlett-Falcone R, Levitzki A, et al. Combination therapy with AG-490 and interleukin 12 achieves greater antitumor effects than either agent alone. Mol Cancer Therapeutics 2002;1:893–9.
25. Blaskovich MA, Sun J, Cantor A, Turkson J, Jove R, Sebti SM. Discovery of JSI-124 (Cucurbitacin I), a selective janus kinase/signal transducer and activitor of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. Cancer Res 2003;63:1270–9.
26. Becker S, Groner B, Muller C. Three-dimensional structure of the Stat3β homodimer bound to DNA. Nature 1998;394:145–50.
27. Jing N, Li Y, Xu X, Li P, Feng L, Tweardy D. Targeting Stat3 with G-quartet oligonucleotides in human cancer cells. DNA Cell Biol 2003;22:685–96.
28. Williamson JR. G-quartet structures in telomeric DNA. Annu Rev Biophys Biomol Structure 1994;23:703–30.
29. Gilber DE, Feigon J. Multistranded DNA structures. Current Opinion Structura Biol 1999;9:305–14.
30. Sen D, Gilbert W. Formation of parallel four-stranded complexes by guanine-rich motif for meiosis. Nature 1998;334:364–6.
31. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 1992;355:564–6.
32. Wyatt JR, Vickers TA, Roberson JL, et al. Combinatorially selected guanosine-quartet structure is a potent inhibitor of HIV envelope-mediated cell fusion. Proc Natl Acad Sci USA 1994;91:1356–60.
33. Rando RF, Ojwang J, Elbaggari A, et al. Suppression of human immunodeficiency virus type 1 activity in vitro by oligonucleotides which form intramolecular tetrads. J Biol Chem 1995;270:1754–60.
34. Mazumder A, Neamati N, Ojwang JO, Sunder S, Rando RF, Pommier Y. Inhibition of the human immunodeficiency virus type 1 integrase by guanosine quartet structures. Biochemistry 1996;35:13762–71.
35. Jing N, De Clercq E, Rando FR, et al. Stability-activity relationships of a family of G-tetrad forming oligonucleotides as potent HIV inhibitors. J Biol Chem 2000;275: 3421–30.
36. Marchand C, Pourquier P, Laco GS, Jing N, Pommier Y. Interaction of human nuclear topoisomerase I with guanosine quartet-forming and guanosine-rich single-stranded DNA and RNA oligonucleotides. J Biol Chem 2002;277:8906–11.
37. Xu X, Hamhouyia F, Thomas SD, et al. Inhibition of DNA replication and induction of —phase cell cycle arrest by G-rich oligonucleotides. J Biol Chem 2001;276: 43221–30.
38. Schreiber WE, Jamani A. Pudek MR. Screening tests for porphobilinogen are insensitive. The problem and its solution. Am J Clin Pathol 1989;92:644–9.
39. Jing N, Hogan ME. Structure-activity of tetrad-forming oligonucleotides as a potent anti-HIV therapeutic drug. J Biol Chem 1998;273:34992–9.
40. Jing N, Xiong W, Guan Y, Pallansch L, Wang S. Potassium dependent folding: a key to intracellular delivery of G-quartet oligonucleotides as HIV inhibitors. Biochemistry 2002;41:5397–403.
41. Bishop JS, Guy-Caffey JK, Ojwang JO, et al. G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. J Biol Chem 1996;271:5698–703.
42. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 2002;109, Suppl:S121–31.
43. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Kuriyan J. Crystal structure of a tyrosine phosphorylated Stat-1 dimer bound to DNA. Cell 1998;93:827–39.
44. Wiederkehr-Adam M, Ernst P, Muller K, et al. Characterization of phosphopeptide motifs specific for the Src homology 2 domains of signal transducer and activator of transcription 1 (STAT1) and STAT3. J Biol Chem 2003;278:16117–28.
45. Wallace TL, Gamba-Vitalo C, Loveday KS, Cossum PA. Acute, multipe-dose, and genetic toxicology of AR177, an anti-HIV oligonucleotide. Toxicol Sci 2000;53:63–70.
46. Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M, Reed JC. Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci USA 1997;94:5113–8.
47. Minn AJ, Velez P, Schendel SL, et al. Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature 1997;385:353–7.

Rational Drug Design of G-Quartet DNA as Anti-Cancer Agents

Naijie Jing*, Wei Sha, Yidong Li, Weijun Xiong and David J. Tweardy

Department of Medicine/Section of Infectious Diseases, Baylor College of Medicine, Houston TX 77030, USA

Abstract: The ability of certain DNA sequences to form G-quartet structures has been exploited recently to develop novel anti-cancer agents including small molecules that promote G-quartet formation within the c-MYC promoter thereby repressing c-MYC transcription and introducing G-quartet-forming oligodeoxynucleotides (GQ-ODN) into cancer cells resulting in p53-dependent cell cycle arrest and inhibition of DNA replication. GQ-ODNs also have been developed as potent inhibitors of signal transducer and activator of transcription (STAT) 3, a critical mediator of oncogenic signaling in many cancers. This review summarizes the rational design of G-quartet forming DNA drugs as Stat3 inhibitors. Topics that are reviewed include the strategy of structure-based drug design, establishment of a structure-activity relationship, development of a novel intracellular delivery system for G-quartet-forming DNA agents and *in vivo* drug testing to assess the anti-cancer effects of DNA drugs in tumor xenografts. Results to date with GQ-ODN targeting Stat3 are encouraging, and it is hoped that continued pursuit of the methodology outlined here may lead to development of an effective agent for treatment of metastatic cancers, such as prostate and breast, in which Stat3 is constitutively activated.

Key Words: DNA drugs, G-quartet oligodeoxynucleotides (GQ-ODN), signal transducer and activator of transcription (STAT) 3, apoptosis, cancer therapy, prostate cancer, breast cancer, drug delivery, drug design.

1. INTRODUCTION

DNA oligonucleotides have been extensively studied as a class of anti-cancer agents and include antisense oligonucleotides, CpG oligonucleotides, and DNA enhancer decoys (see the articles of Cho-Chung [1] and Coppelli and Grandis [2] in this journal issue). Based upon their inhibitory mechanism, DNA-based anti-cancer agents generally belong to one of two categories-antisense or non-antisense. The use of single-stranded antisense oligonucleotides has been extensively reviewed [1, 2]. Upon delivery into cells, these oligonucleotides bind to a target mRNA and function to block its transcription. Non-antisense DNA agents have different mechanisms of action; they interact predominantly with proteins to block their biologic function. In this article, we review G-rich DNA sequences capable of forming G-quartet structures as a new class of non-antisense DNA agents. The inhibitory mechanism of G-quartet oligodeoxynucleotides (GQ-ODNs) involves G-rich DNA forming a stable G-quartet structure, which directly interacts with a target protein to interfere with its function. G-quartet forming DNA has been developed to modulate several biological processes, such as telomerase activity [3], human thrombin activity [4], HIV infection [5, 6] and HIV-1 integrase activity [7-9]. We will summarize the rational design of GQ-ODN as a novel and potentially promising drug for treatment of metastatic tumors in which Stat3 is constitutively activated.

*Address correspondence to this author at the Department of Medicine/ Section of Infectious Diseases, Baylor College of Medicine, Houston TX 77030, USA; Tel: 713-798-3685; Fax: 713-798-8948;
E-mail: njing@bcm.tmc.edu

2. G-QUARTET DNA AS A POTENTIAL ANTI-CANCER AGENT

2.1. Structure and Property of G-Quartet Oligonucleotides

G-rich DNA sequences have been identified, cloned and characterized in the telomeres of many organisms, such as fungi, ciliates, vertebrates, and insects [10]. G-rich sequences can form rigid G-quartet structures, which were first proposed to occur within telomeres [11]. In addition to telomeric sequences [12-14], G-quartet structures have been demonstrated *in vitro* in fragile X syndrome nucleotide repeats [15], in HIV-1 RNA sequences [16, 17], and in the immunoglobulin switch region [18]. G-quartets arise from the association of four G-bases into a cyclic Hoogsteen H-bonding arrangement. Each G-base makes two H-bonds with its neighbor G-base (N1 to O6 and N2 to N7). G-quartets stack on top of each other to give rise to tetrad-helical structures and form a family of structures that include folded G-quartets resulting from intramolecular interactions and hairpin dimers and parallel-stranded tetramers resulting from intermolecular interactions. The formation of G-quartet structures depends on the DNA sequence, loop geometry, base composition of the nucleic acids and the presence of metal ions [19-22]. It was observed that K^+ prefers to induce a folded G-quartet structure and Na^+ preferentially forms a linear four-strand G-quartet structure [23]. The preference of inducing a folded G-quartet structure by metal ions has been proposed as $K^+ > Rb^+ > Na^+ > Li^+$ or Cs^+ [23, 9]. In general, G-quartet structures are very stable; however, their stability depends on the presence of monovalent cations and the concentration of the G-rich oligonucleotides present, especially for dimer or tetramer formation [24-27]. Because of its

1381-6128/05 $50.00+.00

optimal size, potassium interacts within a G-octamer and greatly increases its structural stability [25, 28]. The high concentration of K^+ inside cells (140 mM) significantly promotes the formation by G-rich oligonucleotides of folded G-quartet structures within the cytoplasm [22], greatly increasing their biological activity.

2.2. Designing G-Quartet DNA as an Anti-Cancer Drug

Telomerase has recently become a target for anti-cancer drug therapy. Telomeres shorten with each cell division and cells stop cycling when telomeres shorten below a critical length. The activity of telomerase, which maintains telomere length, is increased in over 85% of cancers examined [29]. Telomeric DNA contains the sequence $(TTAGGG)_n$, which can form a G-quartet structure. The G-quartet structure of this sequence is important in the regulation of telomerase activity [30]. Recently, telomerase has been targeted using DNA oligonucleotides that can serve as telomerase substrates [31, 32]. G-rich oligonucleotides able to form G-quartets were shown to be telomerase inhibitors, [33] and demonstrated inhibitory effects on the growth of tumors cells *in vitro* and in nude mouse tumor xenografts *in vivo* [34]. Also, several small molecules such as BSU 1051 and TMPyP4, were developed using a rational drug design strategy to stabilize G-quartet formation within telomeres, thereby inhibiting the translocation step required for telomerase activity [35-37]. The design of small molecules targeting G-quartet DNA as anti-cancer drugs has recently been well reviewed [38].

2.3. G-Quartet Structures within the Promoter of c-MYC Regulate Its Transcription

Overexpression of the *c*-MYC oncogene occurs in Burkett's lymphoma and in carcinomas such as breast and colon [39-41] where it contributes to increased cellular proliferation and inhibition of differentiation. The nuclease hypersensitivity element (NHE) III within the c-MYC promoter controls 85-90% of c-MYC transcription; Siddiqui-Jain *et al.* [42] identified a G-rich repressor element, Pu27, capable of forming a G-quartet chair structure within NHE III. G-quartet formation by Pu27 is biologically relevant since disruption of G-quartet chair formation results in a 3-fold increase in transcriptional activation of c-MYC. Furthermore, variation of the sequence within the promoter region that alters the ability of intra- and inters molecular G-quartets to form within this region alters transcriptional activation of c-MYC, indicating that G-quartets may comprise on/off gene switches important for the regulation of the c-MYC gene and perhaps other genes as well.

2.4. G-Rich DNA Triggers p53-Dependent Cell Cycle Arrest

It is known that single stranded DNA fragments within mammalian cells can trigger a p53-dependent cell cycle arrest [43]. G-rich single stranded DNA, $(TTAGGG)_2$ capable of forming G-quartets was found to induce a p53-dependent proliferation arrest [44]. Although the mechanism for G-rich ODN activation of p53 is currently unknown, the resulting cell cycle arrest is longer lasting in human fibroblasts than in the telomerase-positive human glioblastoma cell line.

2.5. Interaction of Human Nuclear Topoisomerase I (top I) with G-Quartet DNA

DNA topoisomerases are vital enzymes that control the topological state of DNA by transient cleavage of one (top I) or two strands (top II) of the double helix. Human nuclear top I plays a crucial role in DNA replication, transcription and other cellular functions [45], and has been shown to be the molecular target of many anticancer agents. Topl also has been targeted by designed G-quartet oligonucleotides, such as 16TCC, which form an intermolecular G-quartet structure [46]. Recently, the Pommier group and a collaborator demonstrated that intramolecular G-quartet DNA, such as T30923 (or T30695) [47] and its derivative G-rich ODNs, strongly bond to topl and inhibit its cleavage of duplex DNA with an IC_{50} of ~10 nM [48].

2.6. G-Quartet ODNs have been Designed to Inhibit DNA Replication and Induce S Phase Cell Cycle Arrest

Several non-antisense G-rich ODNs have been reported to be antiproliferative agents, which presumably act by targeting nucleolin or nucleon-like proteins, thus inhibiting the proliferation of tumor cells *in vitro* [49]. Further studies of the effects of G-rich ODN on cell processes found that cells accumulate in S phase in response to treatment with the G-rich ODN, GRO29A, and that GRO29A is capable of inhibiting DNA replication *in vitro*, correlating with its antiproliferative effects [50]. Based on initial studies using UV spectroscopy and circular dichroism (CD), it was proposed that GRO29A forms an anti-parallel G-quartet structure [51]. However, further studies comparing the physical properties and biological activity of GRO29A with a group of 12 GQ-ODNs including the thrombin-binding aptamer, an anti-HIV oligonucleotide, and several quadruplexes derived from telomere sequences, suggest that GRO29A forms an intramolecular G-quartet structure rather than an anti-parallel dimer structure. In these studies, potassium, which stabilizes folded G-quartet structures, was shown to promote the activity of these G-rich ODNs [52].

2.7. Design of G-Quartet ODN to Inhibit Stat3 Activity for Cancer Therapy

Consideration of signal transducer and activator of transcription (STAT) 3 as a target for anti-cancer drug design is based upon the findings that Stat3 is a critical mediator of oncogenic signaling that is activated in 82% of prostate cancers [53], 69% of breast cancers [54], 82-100% of squamous cell carcinoma of the head and neck [55], 71% of nasopharyngeal carcinoma [56], as well as in many other cancers [57-59]. Activated Stat3 up-regulates the expression of anti-apoptotic proteins, such as Bcl-x_L and Mcl-1, thereby decreasing spontaneous apoptosis of cancer cells [60, 61]. Evidence has accumulated that targeting Stat3 and decreasing its activity can block tumor cell growth and enhance apoptosis of cancer cells in which Stat3 is constitutively activated [62-64]. The rationale for targeting Stat3 for cancer therapy also has been reviewed in other articles in this journal issue [2, 65].

We have developed G-quartet oligodeoxynucleotides (GQ-ODNs) as potent inhibitors of Stat3 activity that result in the decreased expression of Stat3-regulated genes, *bcl-x* and Mcl-1, in cancer cells [66]. These agents inhibit the DNA-binding activity of Stat3 preferentially among members of the STAT protein family. Based upon the crystal structure of the Stat3β homodimer and the NMR structure of GQ-ODN [67, 68], a model of GQ-ODN binding to Stat3 homodimer has been constructed that can be exploited for rational drug design. Docking computations resulted in a model in which GQ-ODN insert between the two SH2 domains of the Stat3 homodimer resulting in dimer destabilization. When administered intravenously using a novel drug-delivery system, the GQ-ODN T40214 dramatically inhibited the growth of prostate and breast tumor xenografts in nude mice [69]. Biochemical and histological examination of tumors from mice treated with T40214 demonstrated dramatically reduced expression of anti-apoptotic proteins, Bcl-2 and Bcl-x_L, increased cleavage of caspase 3, and markedly increased apoptosis of tumor cells compared to mice receiving placebo treatment.

3. USE OF A RATIONAL DRUG DESIGN STRATEGY TO DEVELOP GQ-ODN AS A NEW CLASS OF STAT3 INHIBITORS FOR CANCER THERAPY

Rational drug design has become a highly developed technology that uses computational approaches to design or identify new agents for cancer therapy. The design of a new class of molecules that bind to a macromolecular target requires consideration of the type of intermolecular interaction between the inhibitor and target molecule, the geometric relationship between their interacting groups, and the size, shape, and polarity of the inhibitors. In this section, we outline a rational strategy recently used to design a potent GQ-ODN inhibitor of Stat3 activity that integrates structural information with experimental assays to optimize the anti-cancer effects of the inhibitor *in vitro* and *in vivo*.

3.1. Strategy of Rational Drug Design for GQ-ODN

The strategy for rational design of GQ-ODNs as inhibitors of Stat3 activity employed in our laboratory (Fig. **1**) consists of several parts: (i) structure-based drug design, (ii) chemical synthesis, (iii) assays of candidate drug activity *in vitro* and within cells, (iv) rational optimization of drug candidates and (v) assay of *in vivo* drug activity.

3.2. Establishing a Structure-Activity Relationship

A structure-activity relationship (SAR) is a central theme for rational drug design and was a critical step in establishing a potent inhibitor of Stat3 activity. A molecular model of the GQ-ODN/Stat3β complex was first constructed based upon the experimental evidence, which showed that the GQ-ODNs inhibit Stat3 DNA-binding activity by targeting activated Stat3 dimer, not Stat3 monomer, and that treatment of cells with GQ-ODN reduced levels of phosphorylated Stat3, but not total Stat3 within the nucleus [66]. This model also incorporated the results of docking studies using the GRAMM docking program in which the NMR structure of GQ-ODN T30923 [68] was randomly docked onto the crystal structure of the Stat3β dimer [67] two thousand times without setting any binding site restrictions. Analysis of the distribution of H-bonds formed between GQ-ODNs and Stat3β dimer demonstrated that the binding site with the highest binding probability was located within the SH2 domains of each Stat3β from residues 638 to 650 (Fig. **2A**). This region plays a critical role in Stat3 dimerization and was consistent with the experimental observations demonstrating that GQ-ODN destabilized Stat3 dimers [66].

Based upon the molecular model, a structure-activity relationship of GQ-ODN inhibitors was developed [69]. In these studies, we docked three GQ-ODNs, T30923, T40214 and T40216, onto the SH2 domains of the Stat3 dimer one thousand times and analyzed the distribution of H-bonds formed between GQ-ODNs and the residues 638 to 650 within the SH2 domains of Stat3 dimer. Quantitation of the distribution of H-bonds formed between each of the three GQ-ODNs and the Stat3 dimer, especially the percentage of H-bonds that occurred within this critical region, revealed that 27% of the H-bonds formed between T30923 and the Stat3 dimer occurred within this region, 30% of the H-bonds formed by T40216 occurred within this region, and that T40214 had the highest percentage of H-bonds formed in this region with 35% [69]. The percentage of H-bonds formed within the critical region of Stat3 SH2 for each GQ-ODN was directly correlated with its ability to inhibit Stat3 DNA binding activity *in vitro* (IC_{50} of T30923, T40216 and

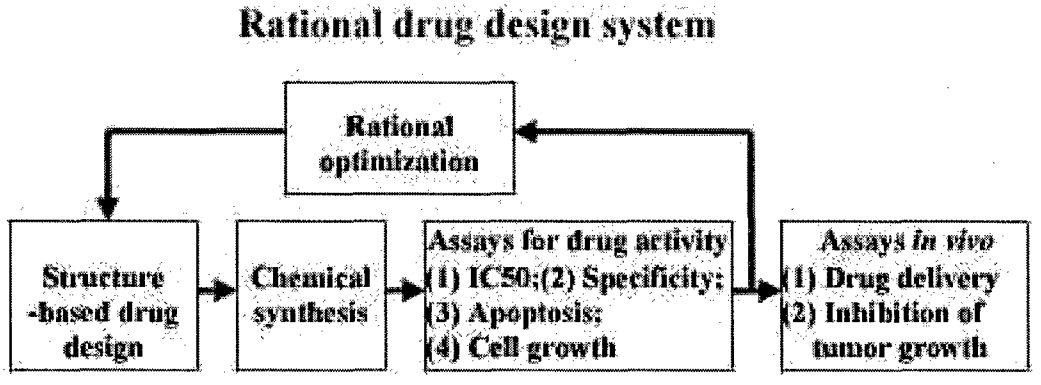


Fig. (1). Scheme of rational drug design for G-quartet DNA drugs.

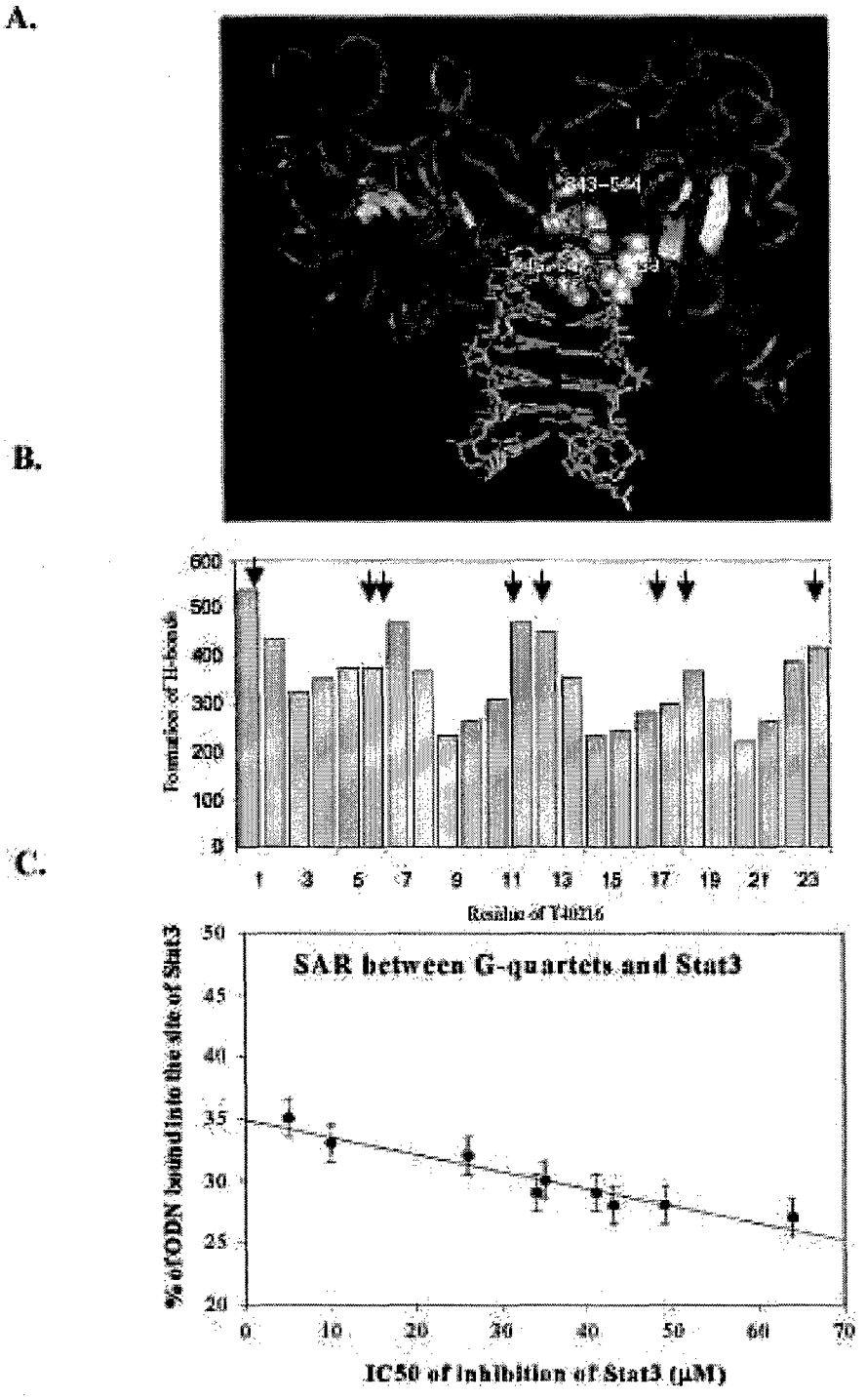


Fig. (2). (A) Model of GQ-ODN T40216 bound to dimers of Stat3 SH2 showing the top-loop domain of T40216 interacting with residues Q643, Q644, N646, N647 and E638 of Stat3 (space-filling model). (B) Histogram of H-bonds for T40216 residues interacting with Stat3. The arrows point to the loop residues of T40216. (C) Plot demonstrating the relationship between the percent of GQ-ODN H-bonding localized from residues 638 to 650 and its IC_{50} against Stat3 [69]. The linear fitting coefficient is 0.91.

T40214 = 25, 12 and 7 μM, respectively [66]). Furthermore, analysis of the histogram of H-bond distribution as a function of residues within GQ-ODN T40216 (Fig. **2B**) showed that the loop residues of GQ-ODNs have the highest probability of interacting with the SH2 domains of the Stat3 dimer. Analogues of the G-quartet ODN T30923 were

designed and synthesized through modification of the NMR structure of GQ-ODN T30923 using the INSIGHTII/ DISCOVER programs. Each analogue was docked onto the SH2 domains of Stat3 dimer one thousand times. Combining the results of the docking analysis with their IC_{50}s revealed a linear correlation between the percentages of GQ-ODN H-binding within the critical site of Stat3 and the IC_{50} of inhibition of Stat3 DNA binding activity (Fig. **2C**), successfully establishing a SAR between GQ-ODNs and the Stat3 dimer [69]. The linear correlation indicates that the higher the percentage of GQ-ODN-Stat3 H-bond formation within the binding site of GQ-ODN to Stat3, the greater it ability to inhibit Stat3. The loop residues of GQ-ODNs are considered to be the most critical, and modifying these residues may improve the potency of the GQ-ODN.

With the establishment of this structure-activity relationship, it is possible to design or screen for a more bioactive GQ-ODN. A GQ-ODN capable of interacting with the Stat3 dimer with a greater percentage of H-bonding within the SH2 domain-binding site would be a good candidate for synthesis and further drug testing.

3.3. Rational Optimization Using Quantitative Structure-Activity Relationship (QSAR)

Rational optimization is an iterative process in which lead compounds are systematically modified to improve their potency and specificity using QSAR. Based upon the model of the GQ-ODN/Stat3 complex, the structure of each designed GQ-ODN was generated and docked onto the SH2 domains of the Stat3 dimer. The binding interaction between the designed GQ-ODN and Stat3 was analyzed. QSAR was developed by combining the docking data of each GQ-ODN with its IC_{50} of inhibition of Stat3. QSAR contains information about several aspects important for designing more potent inhibitors, such as loop structure, length of the G-quartet stem, and exchange of loop residues (Table **1** and Fig. **3**).

(i) The loop structure of GQ-ODN is a critical component that can be modified to improve drug activity. GQ-ODNs T40217, T30923 and T40231, differ in the length of their top-loop structure. Two residues were added to T30923 to form T40217 while two residues were removed to form T40231. The IC_{50} of T40217, T30923 and T40231 determined by electrophoretic mobility shift assay (EMSA) are 64, 25 and 10 μM, respectively. Therefore, decreasing the top-loop residues resulted in increased drug activity.

(ii) Increasing the length of the G-quartet stem caused a decrease in drug activity. GQ-ODN T30923, T40215 and T40216 with two, three and four central G-quartets, were demonstrated to have G-quartet stem lengths of about 15, 19 and 24 Å, respectively [70]. Table **1** shows that IC_{50}s of inhibition of Stat3 DNA-binding activity for T30923, T40215 and T40216 are 25, 49 and 202 μM, respectively, in cancer cells. It is clear that GQ-ODN with longer G-quartet stems have a lower drug activity in cells, suggesting that the size of GQ-ODN is a key factor in determining drug activity. GQ-ODNs with longer stems were also found to enter into cells poorly, corresponding to lower inhibition Stat3 activation [66].

(iii) Substitution of residue T with C in the top-loop domain enhances drug activity. T30923 was determined to have two central G-quartets and two G-T-G-T loops--one each on the top and bottom [68]. The results of CD and non-denatured gels showed that T40214 with two G-C-G-C loops and T40212 with one G-T-G-T loop on top and one G-C-G-C loop on the bottom formed the same G-quartet structures as that of T30923 [71]. IC_{50}s of inhibition of Stat3 DNA-binding activity for T30923, T40212 and T40214 are 25, 35 and 5 μM, respectively. The exchange of residues T with C in the loop domains provides evidence that the top-loop domain of GQ-ODNs, including the residues G1, C8, G9 and C16, is the most critical part of GQ-ODN interacting with Stat3, and suggests that cytosine (C) has a greater binding affinity for Stat3 than thymidine (T). Thus, QSAR results obtained from rational optimization are extremely important to obtain a GQ-ODN with optimal biochemical activity.

Table 1. Relationship Between the Structure of GQ-ODN and Its Inhibition of Stat3 Activation

Oligo	Sequence	IC_{50} (μM)
A) Variation of Loop Structure.		
T40217	GGGGTGGGTGGGTGGGTT	64
T30923	GGGTGGGTGGGTGGGT	25
T40231	GGTGGGTGGGTGGG	10
B) Variation of G-quartet length.		
T30923	$(GGGT)_4$	25
T40215	$(GGGGT)_4$	49
T40216	$(GGGGGT)_4$	202
C) T←→C exchange of loop residues.		
T30923	GGGTGGGTGGGTGGGT	25
T40212	GGGCGGGTGGGCGGGT	35
T40214	GGGCGGGCGGGCGGGC	5

3.4. Design of a GQ-ODN Specifically Targeting Stat3 Among other STAT Protein Members

Specificity of drug action is a critical factor for successful clinical use of a new agent. Specifically targeting Stat3 among other STAT protein members is a highly desirable goal to achieve in designing GQ-ODNs that inhibit cancer cell growth. This sort of specificity is necessary to minimize the potential toxic and immunosuppressive side affects of the drug. Based upon the sequence alignment and known structures, the STAT protein family member that most closely resembles Stat3 is Stat1 [67, 72]. Within cells expressing both STAT proteins, Stat1 heterodimerizes with

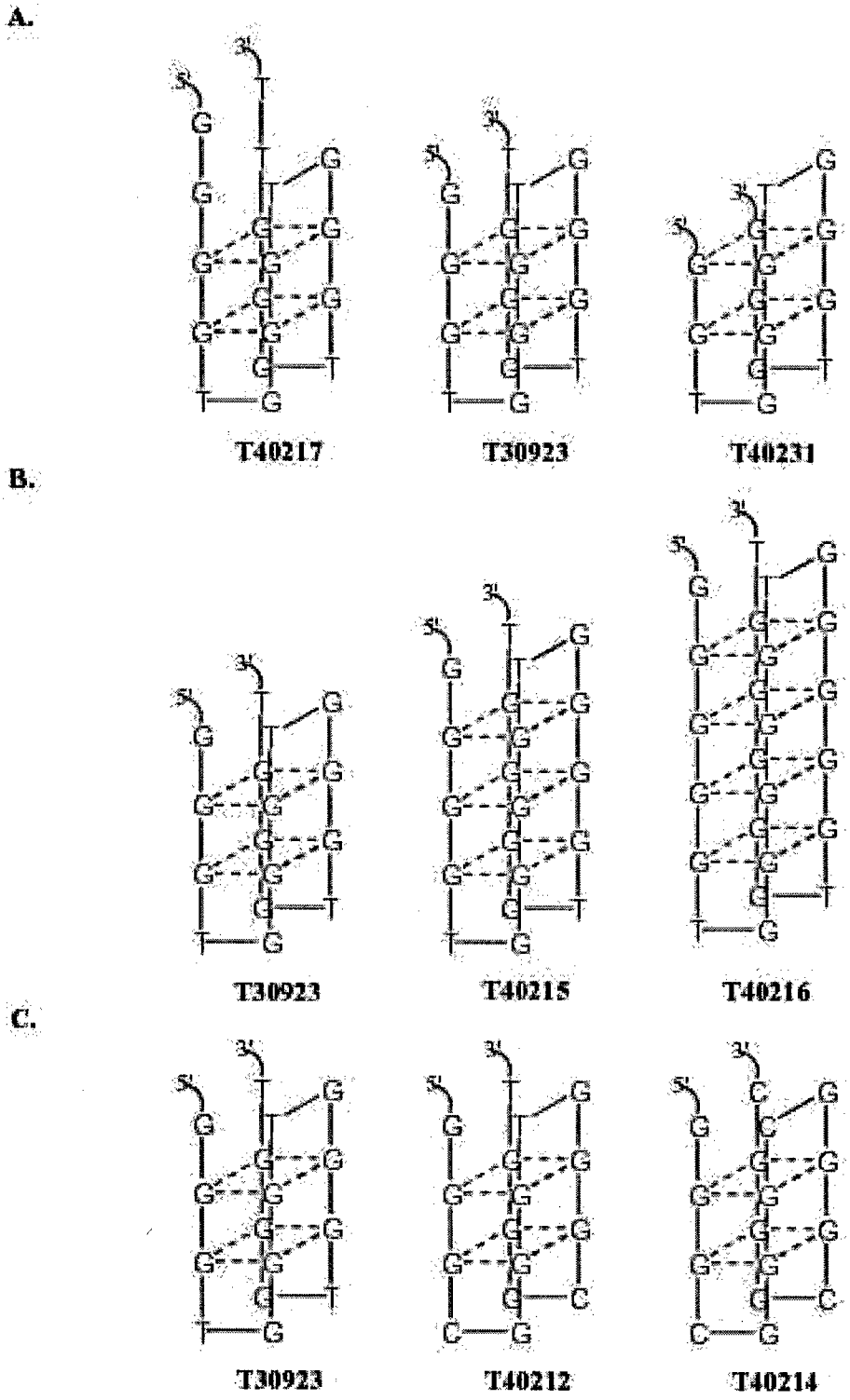


Fig. (3). Schematic structures of G-quartet oligonucleotides.

Stat3 in response to several cytokines including IL-6. Furthermore, Stat3 and Stat1 share more than 50% amino acid sequence homology in the region of their respective SH2 domains. However, in contrast to Stat3, Stat1 has a negative effect on cell proliferation and promotes apoptosis [73]. Therefore, specificity of targeting Stat3 and not Stat1 would have the additional advantage of leaving the natural anti-proliferative and anti-apoptotic effects of Stat1 intact.

To develop a GQ-ODN that inhibits Stat3 and not Stat1, and to gain insight into how a GQ-ODN may preferentially inhibit Stat3 while sparing Stat1, we employed computer-based docking analysis to simulate the interaction between drug and target molecule and to identify the potential binding sites on the target molecule. The GQ-ODN T40214 was randomly docked onto the crystal structure of Stat3β and Stat1 homodimer two thousand times without setting any binding restrictions, as described previously [66]. Docking within the STAT homodimer ranged from amino residues 291 to 716 in one protein and continued from amino residue 716 to 291 in the partner protein. This range encompassed several critical domains including the coiled coil domain, the DNA-binding domain, the linker domain, the SH2 domain and the phosphotyrosine-containing C-terminal tail. Analysis of the histograms of H-bonds formed between the GQ-ODN and the Stat3β or Stat1 homodimer showed that H-bonds formed between GQ-ODN and Stat3 were concentrated within the SH2 and DNA-binding domains. In contrast, H-bonds formed between GQ-ODN and Stat1 were spread out over the whole Stat1 structure [66]. Delivery of the GQ-ODN T40214 into HepG2 cells resulted in 50% inhibition of IL-6-stimulated Stat3 activity at a concentration of 10 μM T40214. In contrast, 50% inhibition of Stat1 was not achieved with concentrations of T40214 up to 142 μM [69]. These results provide solid evidence that GQ-ODN T40214 selectively inhibits activation of Stat3 but not Stat1 in cancer cells [69], and are consistent with the results of our docking studies.

3.5. A Novel Intracellular Delivery System for GQ-ODNs

An effective intracellular delivery system is essential for successful development of DNA drugs as agents to treat cancer. The principal difficulty of delivering GQ-ODNs into cells arises from the physical and structure properties of GQ-ODNs. GQ-ODNs cannot directly penetrate through cell membranes [71]. Furthermore, GQ-ODNs with desirable characteristics, i.e. reduced tendency to aggregate and an overall neutral G-quartet structure, have a reduced capacity to be incorporated into liposomes. The delivery efficiency for lipid/DNA complexes is also inhibited by the presence of serum. However, the inhibitory effect of serum can be overcome by increasing the charge ratio of cationic liposome to DNA. Denaturing the G-quartet structure is required for their incorporation into liposomes. The denatured G-quartet molecules have a high anion charge density, which increases the probability of incorporation with liposomes and decreases the inhibitory effect of serum. A novel intracellular delivery system for GQ-ODNs has been developed (Fig. **4A**) that is based upon the ability of potassium cation to induce a folded G-quartet structure, and exploits the difference in K^+ ion concentration inside (140mM) and outside (4mM) cells. A G-rich ODN, T40214, was designed to form a random coil at low K^+ concentration, which facilitated its incorporation into liposomes, and to form a folded G-quartet structure at higher intracellular K^+ ion concentration, which enabled it to interact with Stat3 and resist endonuclease digestion.

The intracellular delivery of GQ-ODNs is composed of three steps:

(i) *Binding of the lipid/DNA complex onto the cell membranes and uptake by the cell.* Electrostatic interactions are the primary driving force for the binding of the lipid/DNA complex to the cell membrane [74, 75]. The internalization of the lipid/DNA occurs mainly through endocytosis. The main differences in binding efficiency between different lipid/DNA systems are most likely related to their physical properties, such as stability, size and charge density. The charge ratio between cationic lipids and DNA, and the duration of incubation are important parameters for efficient delivery; an increase in the charge ratio and incubation time usually will result in higher delivery efficiency.

(ii) *Release of ODNs into the cytoplasm.* DNA is released into the cytoplasm most likely by disruption of the endosomal membrane, caused by the interaction between cationic lipid and anionic molecules present in the membrane. For various types of liposomes, differences in the frequency of endosomal membrane disruption can occur due to the structure of the hydrophobic part of the cationic lipid. Dioleoyl phosphatidyl ethanolamine (DOPE) is often used as a lipid helper to facilitate disruption of the endosomal membrane. Variation of the ratio of DOPE/liposome affects the percentage and speed of DNA released from lipid/DNA complexes.

(iii) *Penetration of GQ-ODN into the nucleus.* After G-rich ODNs are released into the cytoplasm, they form G-quartet structures induced by the high concentration of K^+ ions inside cells [66, 71]. The highly stable and compact G-quartet structure greatly enhances its ability to resist nuclease digestion and makes it possible for GQ-ODNs to enter the nucleus by passive diffusion through nuclear pores.

The results of the experiments shown in Fig. (**4**) reveal several features of GQ-ODN uptake and activity within cells. After being cultured for 3 hours with G-rich ODNs alone or in a complex with PEI, the cells were washed with fresh medium to remove unbound ODNs or complexes, and then the incubation was continued for 24 hours in fresh, drug-free medium. G-rich ODNs were extracted from the cells and analyzed on a non-denatured gel after the incubation (Fig. **4B**). G-rich ODN T40214 complexed with PEI was released into the cytoplasm and readily formed G-quartet structures (lane 2). In contrast, little T40214 G-quartet formation was detected within extracts of cells incubated with T40214 without PEI (lane 4), indicating that GQ-ODN alone penetrates poorly into cells. Compared with T40214, T30923 has a much lower efficiency of intracellular delivery. Previous studies demonstrated that T30923 forms a G-quartet structure at 5 mM KCl, a condition occurring outside of cells. Thus T30923 formed G-quartet structure before entering the cells, resulting in a decreased intracellular delivery [71]. To assess the impact of G-quartet formation within the cell on Stat3 activity, especially within the nucleus, we isolated nuclear proteins from cancer cells stimulated with IL-6 24 hours after a 3-hr incubation with T40214/PEI complex. The results demonstrated that GQ-ODN T40214 inhibited IL-6-stimulated Stat3 activation in nuclei as the concentration of T40214 was increased from 3.5 to 70 μM (Fig. **4C**).

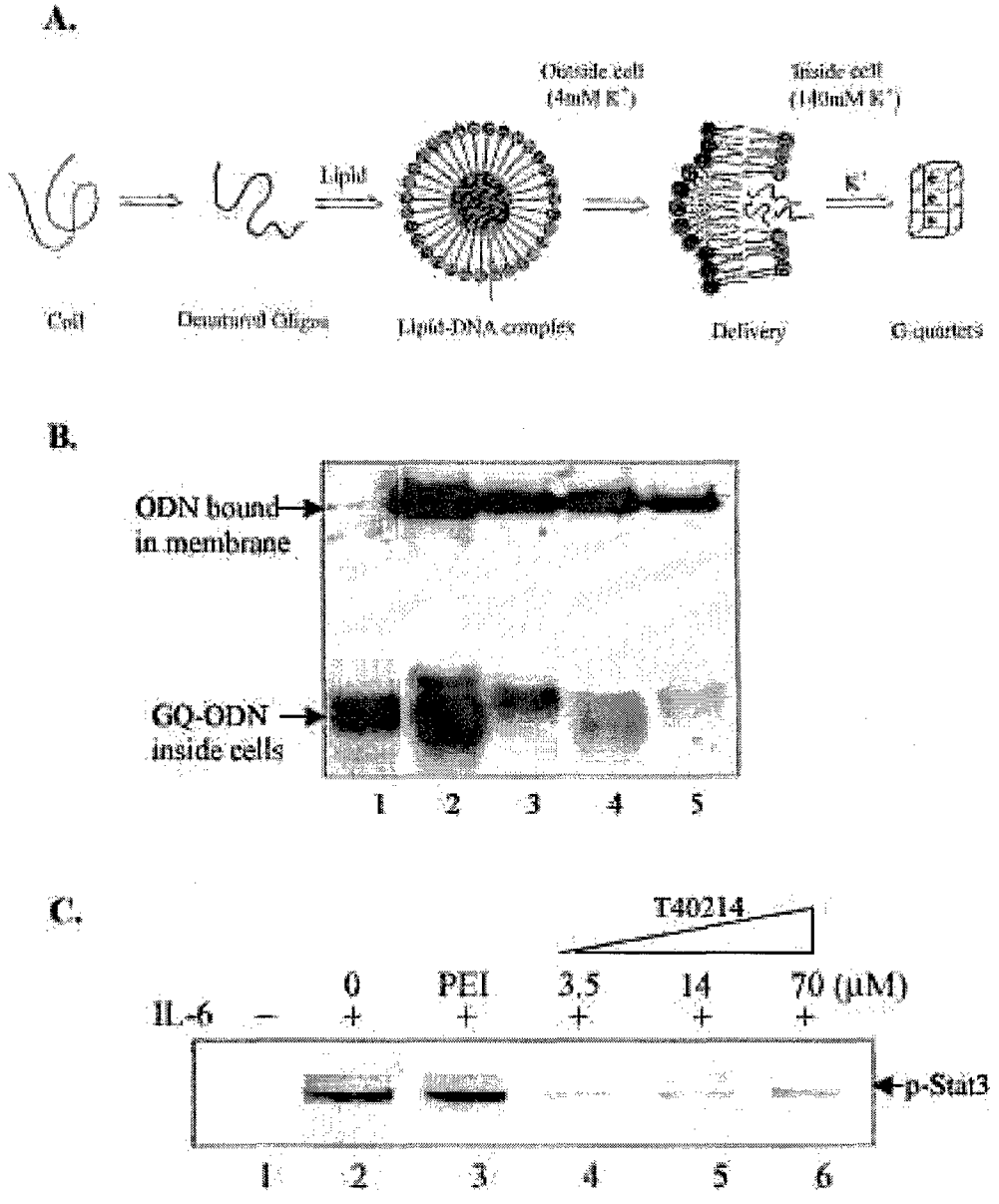


Fig. (4). (A) Scheme of the intracellular delivery system for G-quartet DNA drugs. (B) A non-denatured gel, showing that the molecules of T40214 and T30923 were delivered inside cells 24 hours after PEI/DNA complexes incubated in cells. Lane 1 is free T40214 in the G-quartet structure as a control. Lanes 2 and 4 show T40214 delivered with and without PEI, respectively. Lanes 3 and 5 show T30923 delivered with and without PEI, respectively. (C) Immunoblot of nuclear extracts of HepG2 cells pre-incubated with media (0), PEI alone (PEI) or T40214/PEI at the indicated concentrations for 3 hr. Cells were then washed and incubated in medium alone for 24 hr before stimulation with IL-6 (25 ng/ml); nuclear extracts were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (pY705) of Stat3 (p-Stat3).

3.6. Activity of GQ-ODN is Increased by a Novel Delivery System

To further assess the importance of an efficient delivery system for GQ-ODN activity, we examined the inhibition of IL-6-stimulated Stat3 activation by GQ-ODN T40214 in HepG2 cells 72 hours after cells were incubated with T40214 with or without PEI [69]. In this experiment, HepG2 cells were incubated for 3 hrs with T40214 with or without PEI, washed twice with fresh medium to remove unbound DNA/PEI complex, incubated in medium without T40214/PEI for 72 hours, then stimulated with IL-6 for 30 min. EMSA of cell extracts demonstrated that incubation with T40214/PEI resulted in 50% inhibition of Stat3 activation at a T40214 concentration of 10 μM. In contrast, no inhibition of Stat3 activation was observed in extracts of cells incubated in T40214 alone at concentrations as high as 142

μM, confirming our previous observation that GQ-ODN alone without carrier has low biological activity.

RNAse protection assays (RPA) were also performed on RNA from cells incubated with T40214/PEI to examine their effect on mRNA levels of Stat3-regulated genes such as Bcl-x_L and Mcl-1. Bcl-x_L is an anti-apoptotic protein within the Bcl-2 family, and is the major protein translated from the *bcl-x* gene [76-78]. Mcl-1 also represents a survival factor for human cancer cells [79]. The results of pre-incubation of HepG2 cells with T40214/PEI complexes demonstrated that Bcl-x mRNA was completely inhibited by T40214 at 50ng/μl and Mcl-1 mRNA was suppressed about 50% at the same concentration of T40214 [66].

3.7. Delivery of GQ-ODN *In vivo*

To determine if GQ-ODNs can be effectively delivered into cells *in vivo*, fluorescent labeled T40214 plus PEI was injected into the tail vein of male and female mice and rats. Twenty-four hours after infusion, animals were sacrificed. The organs were harvested, frozen, microtome sectioned and examined by fluorescence microscopy. Intracellular fluorescence indicative of GQ-ODN T40214 uptake was observed in all tissues examined including the prostate gland and breast of mice and rats (Fig. **5A**). Importantly, fluorescence was readily detected within tumor xenografts isolated from nude mice [69], indicating that GQ-ODN enter their cancer cell targets when administered intravenously with PEI.

3.8. *In vivo* Tests for drug Candidates

Examination of the effectiveness of a drug in animal models is an important step on the way to establishing its potential clinical utility. Nude mice with tumor xenografts were used as an animal model for *in vivo* drug testing to evaluate the potential of GQ-ODNs as anticancer agents. Nude mice were injected subcutaneously with human cancer cell lines, such as prostate (PC-3) and breast (MDA-MB-468), in which Stat3 is constitutively active. When tumors were established, mice were randomly assigned into two groups. One group (drug-treated) received T40214/PEI complexes and the other group (placebo) received PEI alone every other day. The results demonstrated that the mean size of the prostate tumor xenografts of placebo-treated mice increased by 9 fold while that of drug-treated mice increased by only 2.2 fold ($p=0.001$). Similarly, the mean size of the breast tumor xenografts of placebo-treated mice increased 6.7 fold while that of drug-treated mice remained unchanged ($p=0.006$) [69]. The statistic t-test calculation was also performed to determine the tumor growth rates, using the function $[t=(X_1-X_2)/S_p^2(1/n_1+1/n_2))^{1/2}]$, where X_1 and X_2 are the average values of tumor sizes of the placebo and drug-treated groups, respectively, and S_p^2 is a pooled variance. The results showed that the average rates of prostate tumor growth (PC-3) for placebo-treated and drug-treated mice were increased by 23%/day and 10%/day, respectively, and that the average rates of breast tumor growth (MDA-MB-468) for placebo-treated and drug-treated mice were increased by in 13%/day and decreased by 0.4%/day, respectively. This 5 mg/kg dose of GQ-ODN T40214 suppressed tumor growth rate by more than 10%/day compared with the rates for placebo mice. T40214 had a dramatic *in vivo* inhibitory effect on the growth of established prostate and breast cancers in nude mice when administered by intravenous injection, and shows promise as a therapeutic agent for these cancers in humans (Fig. **5B**).

3.9. GQ-ODN is a New Class of Anti-Cancer Agent

To determine the mechanism of inhibition of tumor xenograft growth by T40214, we harvested the prostate tumors from drug- and placebo-treated mice after 10 days of treatment and extracted proteins to assess levels of activated Stat3 protein (Stat3 phosphorylated on Y705, p-Stat3), Bcl-x_L and Bcl-2 proteins, and activated caspase 3. In xenografts from drug-treated animals, levels of p-Stat3, Bcl-x_L and Bcl-2 decreased 9, 4 and 10 fold, respectively, while caspase 3 cleavage products increased 3-fold compared to xenografts from placebo-treated mice. Deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) was performed as an assay for apoptosis and revealed an 8-fold increase in apoptotic cells within xenografts from drug-treated mice compared to xenografts from placebo-treated mice [69].

The growth inhibitory effects of GQ-ODN T40214 are mediated through inhibition of Stat3 activation and reduction of Bcl-2 and Bcl-x_L protein levels. Bcl-2 and Bcl-x_L, which have ion channel activity, have been reported to inhibit release of cytochrome C from mitochondria [80, 81]. Blocking the expression of Bcl-2 and Bcl-x_L can increase the release of cytochrome C, resulting in activation of the caspase cascade, which includes caspase 3, leading to apoptosis.

3.10. Potential for Increasing the Anti-Tumor Activity of GQ-ODN

The effect of GQ-ODNs on tumor suppression strongly depends on the difference between the rates of cancer cell growth and cell death. In the GQ-ODN-treated PC-3 prostate tumors, cell growth presumably is faster than cell death; while in GQ-ODN-treated MDA-MB-468 breast tumors, the rate of tumor cell growth is equal to the rate of cell apoptosis. Several factors may influence the drug activity level *in vivo* including differences in expression and effects of Stat3 in each tumor, and differences in the concentration of drug required to enter into a tumor cell and inhibit Stat3. Therefore, it is important to identify the optimum conditions for treatment of each human cancer, including the necessary drug concentration, the ratio of PEI/ODN and the interval time between treatments.

4. SUMMARY

4.1. Mechanism of GQ-ODN as an Inhibitor of Stat3

We modeled the inhibition of Stat3 by GQ-ODNs as shown in Fig. **6**. Monomers of Stat3 in the cytoplasm of unstimulated cells become activated by cytokines or growth factor receptors, including IL-6 and EGFR. Stat 3 is also recruited by intrinsic or receptor-associated tyrosine kinases, such as JAK. Tyrosine phosphorylation of Stat3 induces formation of active Stat3 dimers via its SH2 domains, and the activated Stat3 dimers translocate to the nucleus, where they bind to DNA-response elements in the promoters of target genes and activate specific gene expression programs

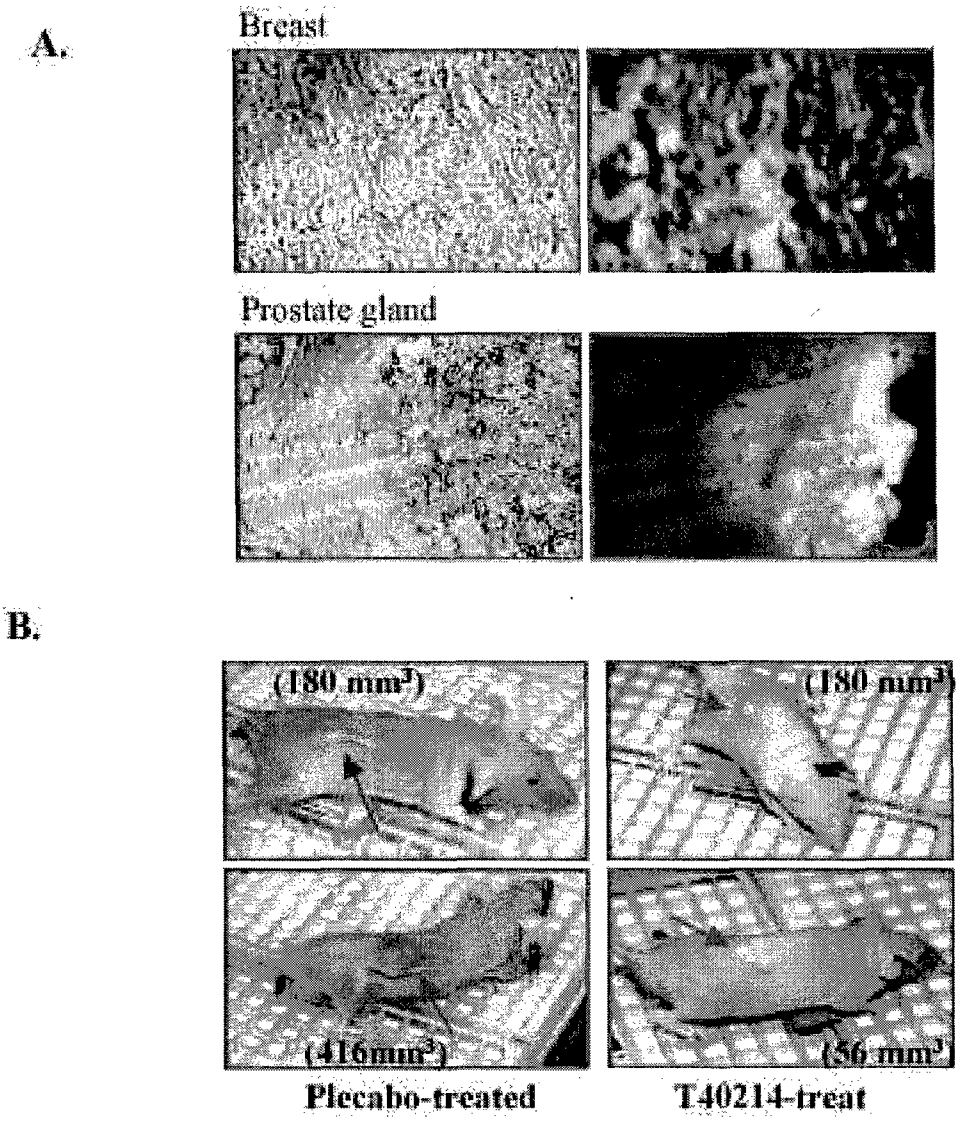


Fig. (5). (A) Non-fluorescent micrographs (200x magnification) (left panels) show the tissues of breast and prostate gland and fluorescent micrographs (right panels) show the distribution of labeled T40214 in the same tissues of breast and prostate gland. (B) *In vivo* drug tests by intravenous injection of T40214 after establishing prostate tumors (PC-3) on nude mouse xenografts. The injections were performed every two days with T40214 (5mg/kg) plus PEI (2.5mg/kg) as drug treatments and with PEI alone as placebo treatments. Photographs of representative mice at the start of treatment (upper panels) and on day 11 after five treatments (lower panels).

[57-59]. GQ-ODNs are delivered into the cytoplasm by PEI/DNA complexes. Induced by the elevated K^+ concentration within the cytoplasm, ODNs form G-quartet structures, which diffuse into the nucleus and inhibit activation of phosphorylated Stat3. The inhibition of Stat3 activation decreases expression of Stat3-regulated genes, notably those encoding anti-apoptotic proteins, such as Bcl-x_L, Bcl-2 and Mcl-1, which reduces their levels below a critical threshold and triggers cell apoptosis.

4.2. Rational Design of G-Quartet DNA Drugs

A rational drug design system involves successive rounds of construction of a model for the inhibitor-target complex, the search for a lead compound using molecular modeling tools, synthesis of new drug candidates, and measurement of physical and biological properties of the modified candidates. Rational optimization can increase the success rate for a preclinical candidate and enable the prediction of their drug properties such as efficacy, toxicity and side effects. Also, optimization can make a backup rapidly available when a candidate is found to have inadequate drug properties in clinical trials.

A drug design system is based upon two assumptions: (i) The structure of the target molecule contains a binding site with a reasonably defined molecular shape, and (ii) the structure of the inhibitor/target molecule complex obtained

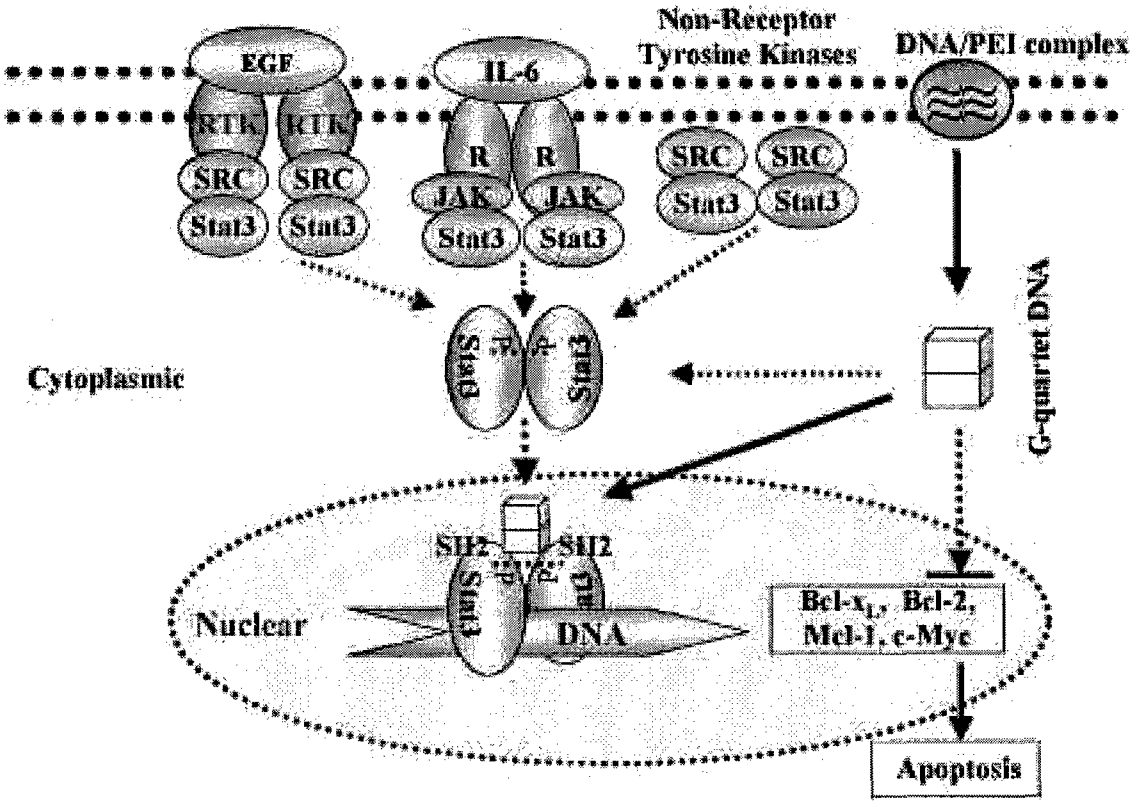


Fig. (6). Scheme of the mechanism of inhibition of Stat3 by GQ-ODNs.

from docking is a useful starting point for searching for an effective inhibitor. There are several docking programs available for drug design, such as INSIGHTII/DOCK, AUTODOK, and GRAMM. To construct a drug design system, we employed a statistical docking system using GRAMM and randomly docked the known structure of GQ-ODN into the crystal structure of the Stat3β dimer one or two thousand times without setting binding site restrictions. The results of the statistical docking demonstrated the probability of an interaction between drug and target molecule, identified the potential binding sites on the target molecule, and established a structure of the GQ-ODN/Stat3β dimer complex for our structure-based drug design [66].

Structure-activity relationship (SAR) has proven to be extremely useful in the design of biologically active molecules. SAR correlates the relative biological potency of inhibitors with their physical properties. Establishing a specific SAR is a key step for each drug design system. For example, the SAR of G-quartet DNA oligodeoxynucleotides as inhibitors of HIV-1 integrase has been established previously [24] The stability-activity correlation demonstrated that the structural stability of G-quartet DNA oligodeoxynucleotides is an important determinant for inhibition of HIV-1 integrase activity *in vitro* and in cells. As another example, a linear SAR was recently established that directly correlated the ability of a GQ-ODN to bind to a region within the Stat3 SH2 domain, which is critical for dimerization, with its ability to inhibit Stat3 activity within cells [69]. The correlation indicated that the higher the percentage of GQ-ODN H-bond formation within this region of Stat3 SH2, the greater its ability to inhibit Stat3 activation within cells. This is an important step towards optimizing the design of GQ-ODN inhibitors of Stat3.

4.3. Advantages of G-Quartet DNA Drugs

GQ-ODNs as DNA drugs for cancer therapy have several novel and important features. G-rich ODNs with a stable G-quartet structure directly interact with target molecules, functioning in a manner similar to small molecule inhibitors. Large active sites are difficult to target using small molecular inhibitor approaches. However, the size of the G-quartet structure makes it possible to block target molecules with large active sites such as the SH2 domains of Stat3 homodimers. Thus, GQ-ODN can be considered a complementary approach to small molecular inhibitors targeting molecules with large active sites, such as Stat3. The intramolecular G-quartet structure prevents single-strand endonucleases from accessing their cleavage sites, leading to a long oligonucleotide half-life in serum and inside cells [82]. Also, G-quartet ODNs demonstrate low toxicity. Toxicity studies have been reported for a GQ-ODN, T30177 (AR177), as an inhibitor of HIV-1 integrase [83]. T30177 did not exhibit genetic toxicity in three different mutagenic assays: *Ames*/Salmonella mutagenesis assay, CHO/HGPRT mammalian cell mutagenesis assay, and mouse micronucleus assay. Acute toxicity studies in mice showed that the GQ-ODN T30177 has an LD50 (dose lethal to 50% of treated animals) at greater than or equal to 1.5g/kg body weight. Multiple dose toxicity studies

in mice showed that T30177 did not cause male-specific mortality or changes in serum chemistry, hematology, and histology until doses reached 250 and 600 mg/kg, which are more than 100-fold that of the therapeutic dose. Hence, structure-based drug design of GQ-ODNs increases the likelihood of designing a potent drug with better specificity and a lower toxicity profile.

CONCLUSION

A novel inhibitor of Stat3 will be helpful both as a single agent treatment, and as part of combination therapy. Current treatments for androgen-independent prostate cancer have not shown a definitive increase in survival [84]. The treatment options employed for patients with advanced and metastatic cancer, such as surgery, radiation, hormone therapy and chemotherapy, are limited [85]. Metastatic breast cancer also remains incurable using the currently available chemotherapeutic treatments. G-quartet DNA targeting Stat3 induces tumor cell apoptosis when delivered to tumor xenografts following intravenous injection. Therefore, the G-quartet DNA drugs represent a novel class of therapeutic agents that hold promise for the systemic treatment of prostate and breast cancers, particularly since Stat3 is activated in 82% of prostate cancer cells [53] and 70% of breast cancer cells [54]. A G-quartet DNA could also be a powerful non-antisense agent for other human cancers where Stat3 is active.

ACKNOWLEDGEMENT

This work was supported by DOD award PC020407 and, in part, by R01 CA86430.

REFERENCES

[1] Cho-Chung YS. DNA drug design for cancer therapy. Curr Pharm Design 2004; (this issue).

[2] Coppelli, F. M. and Grandis, J. R. Oligonucleotides as anticancer agents: from the benchside to the clinical and beyond. Curr Pharm Design 2004; (this issue).

[3] Sen D, Gilbert W. Formation of parallel four-stranded complexes by guanine-rich motif for meiosis. Nature 1998; 334: 364-6.

[4] Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 1992; 355: 564-6.

[5] Marshall WS, Beaton G, Stein CA, Matsukura M, Caruthers MH. Inhibition of HIV activity by phosphodithioate oligonucleoxytidine. Proc Natl Acad Sci USA 1992; 89: 6256-9.

[6] Wyatt JR, Vickers TA, Roberson JL, Buckeit RW, Klimkait T, Debaets E, *et al.* Combinatorially selected guanosine-quartet structure is a potent inhibitor of HIV envelope-mediated cell fusion. Proc Natl Acad Sci USA 1994; 91: 1356-60.

[7] Rando RF, Ojwang J, Elbaggari A, Reyes GR, Tinder R, McGrath MS, *et al.* Suppression of human immunodeficiency virus type 1 activity *in vitro* by oligonucleotides which form intramolecular tetrads. J Biol Chem 1995; 270: 1754-60.

[8] Mazumder A, Neamati N, Ojwang JO, Sunder S, Rando RF, Pommier Y. Inhibition of the human immunodeficiency virus type 1 integrase by guanosine quartet structures. Biochemistry 1996; 35: 13762-71.

[9] Jing N, Rando FR, Pommier Y, Hogan ME. Ion selective folding of loop domains in a potent anti-HIV oligonucleotide. Biochemistry 1997; 36: 12498-505.

[10] Henderson E. Telomere DNA structure. Telomeres, Cold Spring Harbor Laboratory Press 1995; 11-34.

[11] Gellert M, Lipsett MN, Davies DR. Helix formation by guanylic acid. Proc Natl Acad Sci USA1962; 48: 2013-18.

[12] Sundquist WI, Klug A. Telomeric DNA dimerizes by formation of guanine tetrads between hairpin loops. Nature 1989; 342: 825-8.

[13] Smith FW, Feigon J. Quadruplex structure of Oxytricha telomeric DNA oligonucleotides. Nature 1992; 356: 164-8.

[14] Kang CH, Zhang X, Ratliff R, Moyzis R, Rich A. Crystal structure of four-stranded Oxytricha telomeric DNA. Nature 1992; 356: 126-31.

[15] Fry M, Leob LA. The fragile X syndrome d(CGG)n nucleotide repeats form a stable tetrahelical structure. Proc Natl Acad Sci USA 1994; 91: 4950-4.

[16] Sundquist WI, Heaphy S. Evidence for interstrand quadruplex formation in the dimerization of human immunodeficiency virus 1 genomic RNA. Proc Natl Acad Sci USA 1993; 90: 3393-7.

[17] Awang G, Sen D. Mode of dimerization of HIV-1 genomic RNA. Biochemistry 1993; 32: 11453-7.

[18] Sen D, Gilbert W. Formation of parallel four-stranded complexes by guanine-rich motif for meiosis. Nature 1998; 334: 364-6.

[19] Williamson JR. G-quartet structures in telomeric DNA. Annu Rev Biophys Biomol Struct 1994; 23: 703-30.

[20] Gilber DE, Feigon J. Multistranded DNA structures. Curr Opin Struct Biol 1999; 9: 305-14.

[21] Rhodes R, Giraldo R. Telomere structure and function. Curr Opin Struct Biol 1995; 5: 311-22.

[22] Shafer RH, Smirnov I. Biological aspects of DNA/RNA quadruplexes. Bioplolymers 2001; 56: 209-27.

[23] Sen D, Gillbert W. A sodium-potassium switch in the formation of four-stranded G4-DNA. Nature 1990: 344: 410-4.

[24] Jing N, De Clercq E, Rando FR, Pallansch L, Lackman-Smith C, Lee S, *et al.* Stability-activity relationships of a family of G-tetrad forming oligonucleotides as potent HIV inhibitors. J Biol Chem 2000; 275: 3421-30.

[25] Pilch DS, Plum GE, Breslauer KJ. The thermodynamics of DNA structures that contain lesions or guanine tetrads. Curr Opin Struct Biol 1995; 5: 334-42.

[26] Mergny JL, Phan AT, Lacroix L. Following G-quartet formation by UV-spectroscopy. FEBS Lett 1998; 435: 74-8.

[27] Gou Q, Lu M, Kallenbach NR. Effect of thymine tract length on the structure and stability of model telomeric sequences. Biochemistry 1993; 32: 3596-603.

[28] Han H, Hurley LH. G-quadruplex DNA: a potential target for anticancer drug design. Trends Pharmacol Sci 2000; 21: 136-42.

[29] Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, *et al.* Specific association of human telomerase activity with immortal cells and cancer. Science 1995; 268: 1115-7.

[30] Mata JE, Joshi SS, Palen B, Pirruccello SJ, Jackson JD, Elias N, *et al.* A hexameric phosphorothioate oligonucleotide telomerase inhibitor arrests growth of Burkitt's lymphoma cells *in vitro* and *in vivo.* Toxicol Appl Pharmacol 1997; 144: 189-97.

[31] Raymond E, Soria JC, Izbicka E, Boussin F, Hurley L, Von Hoff DD. DNA G-quadruplexes, telomere-specific proteins and telomere-associated enzymes as potential targets for new anticancer drugs. Invest New Drugs 2000; 18: 123-37.

[32] Sun D, Lopez-Guajardo CC, Quada J, Hurley LH, Von Hoff DD. Regulation of catalytic activity and processivity of human telomerase. Biochemistry 1999; 38: 4037-44.

[33] Mata JE, Joshi SS, Palen B, Pirruccello SJ, Jackson JD, Elias N, *et al.* A hexameric phosphorothioate oligonucleotide telomerase inhibitor arrests growth of Burkitt's lymphoma cells *in vitro* and *in vivo.* Toxicol Appl Pharmacol 1997; 144: 189-97.

[34] Kondo S, Kondo Y, Li G, Silverman RH, Cowell JK. Targeted therapy of human malignant glioma in a mouse model by 2-5A antisense directed against telomerase RNA. Oncogene 1998; 16: 3323-30.

[35] Perry PJ, Reszka AP, Wood AA, Read MA, Gowan SM, Dosanjh HS, *et al.* Human telomerase inhibition by regioisomeric disubstituted amidoanthracene-9,10-diones. J Med Chem 1998; 41: 4873-84.

[36] Perry PJ, Gowan SM, Reszka AP, Polucci P, Jenkins TC, Kelland LR, *et al.* 1,4- and 2,6-disubstituted amidoanthracene-9,10-dione derivatives as inhibitors of human telomerase. J Med Chem 1998; 41: 3253-60.

[37] Izbicka E, Wheelhouse RT, Raymond E, Davidson KK, Lawrence RA, Sun D, *et al.* Effects of cationic porphyrins as G-quadruplex

interactive agents in human tumor cells. Cancer Res 1999; 59: 639-44.

[38] Hurley LH, Wheelhouse RT, Sun D, Kerwin SM, Salazar M, Fedoroff OY, *et al.* G-quadruplexes as targets for drug design. Pharmacol Ther 2000; 85: 141-58.

[39] Spencer CA, Groudine M. Control of c-myc regulation in normal and neoplastic cells. Adv Cancer Res 1991; 56: 1-48.

[40] Marcu KB, Bossone SA, Patel AJ. myc function and regulation. Annu Rev Biochem 1992; 61: 809-60.

[41] Facchini LM, Penn LZ. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J 1998; 12: 633-51.

[42] Siddiqui-Jain A, Grand CL, Bearss DJ, Hurley LH. Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. Proc Natl Acad Sci USA 2002; 99: 11593-8.

[43] Huang LC, Clarkin KC, Wahl GM. Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. Proc Natl Acad Sci USA 1996; 93: 4827-32.

[44] Saretzki G, Sitte N, Merkel U, Wurm RE, von Zglinicki T. Telomere shortening triggers a p53-dependent cell cycle arrest via accumulation of G-rich single stranded DNA fragments. Oncogene 1999; 18: 5148-58.

[45] Wang, JC. Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 2002; 3: 430-40.

[46] Arimondo, PB, Riou J-F, Mergy J-L, Tazi J, Sun J-S, Garestier T, *et al.* Interaction of human DNA topoisomerase I with G-quartet structures. Nucleic Acids Res 2000; 28: 4832-8.

[47] Jing N, Marchand C, Liu J, Mitra R, Hogan ME, Pommier Y. Mechanism of inhibition of HIV-1 integrase by G-tetrad-forming oligonucleotides *in vitro.* J Biol Chem 2000; 275: 21460-7.

[48] Marchand C, Pourquier P, Laco GS, Jing N, Pommier Y. Interaction of human topoisomerase I with Guanosine quartet-forming and Guanosine-rich single-stranded DNA and RNA oligonucleotides. J Biol Chem 2002; 277: 8906-11.

[49] Bates PJ, Kahlon JB, Thomas SD, Trent JO, Miller DM. Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. J Biol Chem 1999; 274: 26369-77.

[50] Xu X, Hamhouyia F, Thomas SD, Burker TJ, Girvan AC, Glenn McGregor W, *et al.* Inhibition of DNA replication and induction of Sphase cell cycle arrest by G-rich oligonucleotides. J Biol Chem 2001; 276: 43221-30.

[51] Dapic V, Bates PJ, Trent JO, Rodger A, Thomas SD, Miller DM. Antiproliferative activity of G-quartet-forming oligonucleotides with backbone and sugar modifications. Biochemistry 2002; 41: 3676-85.

[52] Dapic V, Abdomerovic V, Marrington R, Peberdy J, Rodger A, Trent JO, *et al.* Biophysical and biological properties of quadruplex oligodeoxyribonucleotides. Nucleic Acids Res 2003; 31: 2097-107.

[53] Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, *et al.* Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002; 62: 6659-66.

[54] Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL, Rimm DL. Tissue microarray analysis of signal transducers and activatior of transcription 3 and phospho-state 3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clin Cancer Res 2003; 9: 594-600.

[55] Nagpal JK, Mishra R, Das BR. Activation of Stat3 as one of early events in tobacco chewing-mediated oral carcinogenesis. Am Can Soc 94: 2393-400.

[56] Hsiao J-R, Jin Y-T, Tsai T, Shiau A-L, Wu C-L, Su W-C. Constitutive activation of Stat3 and Stat5 is present in majority of nasopharyngeal carcinoma and correlates with better prognosis. Br J Cancer 2003; 89: 344-9.

[57] Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000; 19: 2474-88.

[58] Bromber JF. Activation of STAT proteins and growth control. BioEssay 2001; 23: 161-9.

[59] Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumor provides novel molecular targets for therapeutic intervention. Clin Cancer Res 2002; 8: 945-54.

[60] Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, *et al.* Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74: 597-608.

[61] Zhou P, Qian L, Kozopas KM, Craig RW. Mcl-1, a bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 1997; 89: 630-43.

[62] Grandis JR, Drenning SD, Zeng Q, Watkins SC, Melhem MF, Endo S, *et al.* Constitutive activation of stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. Proc Natl Acad Sci USA 2000; 97: 4227-32.

[63] Burdelya L, Catlett-Falcone R, Levitzki A, Cheng F, Mora LB, Sotomayor E, *et al.* Combination therapy with AG-490 and interleukin 12 achieves greater antitumor effects than either agent alone. Mol Cancer Therapeut 2002; 1: 893-9.

[64] Blaskovich MA, Sun J, Cantor A, Turkson J, Jove R, Sebti SM. Discovery of JSI-124 (Cucurbitacin I), a selective janus kinase/signal transducer and activitor of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. Cancer Res 2003; 63: 1270-9.

[65] Redell MS, Tweardy DJ. Targeting transcription factors for cancer therapy. Curr Pharm Design 2004; (this issue).

[66] Jing N, Li Y, Xu X, Li P, Feng L, Tweardy D. Targeting Stat3 with G-quartet oligonucleotides in human cancer cells. DNA Cell Biol 2003; 22: 685-96.

[67] Becker S, Groner B, Muller C. Three-dimensional structure of the Stat3β homodimer bound to DNA. Nature 1998; 394: 145-50.

[68] Jing N, Hogan ME. Structur-activity of tetrad-forming oligonucleotides as a potent anti-Hiv therapeutic drug. J Biol Chem 1998; 273: 34992-9.

[69] Jing N, Li Y, Xiong W, Sha W, Jing L, Tweardy DJ. G-quartet oligonucleotides: a new class of Stat3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. Cancer Res 2004; 64: 6603-6609.

[70] Jing N, Marchand C, Liu J, Mitra R, Hogan ME, Pommier Y. Mechanism of Inhibition of HIV-1 Integrase by G-tetrad Forming Oligonucleotides *in vitro.* J Biol Chem 2000; 275(28): 21460-7.

[71] Jing N, Xiong W, Guan Y, Pallansch L, Wang S. Potassium dependent folding: a key to intracellular delivery of G-quartet oligonucleotides as HIV inhibitors. Biochemistry 2002; 41(17): 5397-403.

[72] Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Kuriyan J. Crystal structure of a tyrosine phosphorylated Stat-1 dimer bound to DNA. Cell 1998; 93: 827-39.

[73] O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 2002; 109 Suppl: S121-31.

[74] Maurer N, Mori A, Palmer L, Monck MA, Mok KWC, Mui B, *et al.* Lipid-based systems for the intracellular delivery of genetic drugs. Mol Membrane Biol 1999; 16: 129-40.

[75] Chesnoy S, Huang L. Structure and function of lipid-DNA complexes for gene delivery. Annu Rev Biophys Biomol Struct 2000; 29: 27-47.

[76] Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, *et al.* Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74: 597-608.

[77] Gonzalez-Garcia M, Perez-Ballestero R, Ding L, Duan L, Boise LH, Thompson CB, *et al.* Bcl-x_L is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development 1994; 120: 3033-42.

[78] Grad JM, Zeng XR, Boise LH. Regulation of Bcl-x_L: a little bit to this and a little bit to STAT. Curr Opin Oncol 2001; 12: 543-49.

[79] Zhou P, Qian L, Kozopas KM, Craig RW. Mcl-1, a bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 1997; 89: 630-43.

[80] Schendel S,L, Xie Z, Montal MO, Matsuyama S, Montal M, Reed JC. Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci USA 1997; 94: 5113-8.

[81] Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, *et al.* Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature 1997; 385: 353-7.

[82] Bishop JS, Guy-Caffey JK, Ojwang JO, Smith SR, Hogan ME, Cossum PA, *et al.* G-quartet motifs confer nuclease resistance to a

potent anti-HIV oligonucleotide. J Biol Chem 1996; 271: 5698-703.

[83] Wallace TL, Gamba-Vitalo C, Loveday KS, Cossum PA. Acute, multipe-dose, and genetic toxicology of AR177, an anti-HIV oligonucleotide. Toxicol Sci 2000; 53: 63-70.

[84] Gulley J, Dahut W. Novel clinical trials in androgen-independent prostate cancer. Clin Prostate Cancer 2002; 1: 51-7.

[85] Benimetskaya L, Stein CA. Antisense therapy: recent advances and relevance to prostate cancer. Clin Prostate Cancer 2002; 1: 20-30.